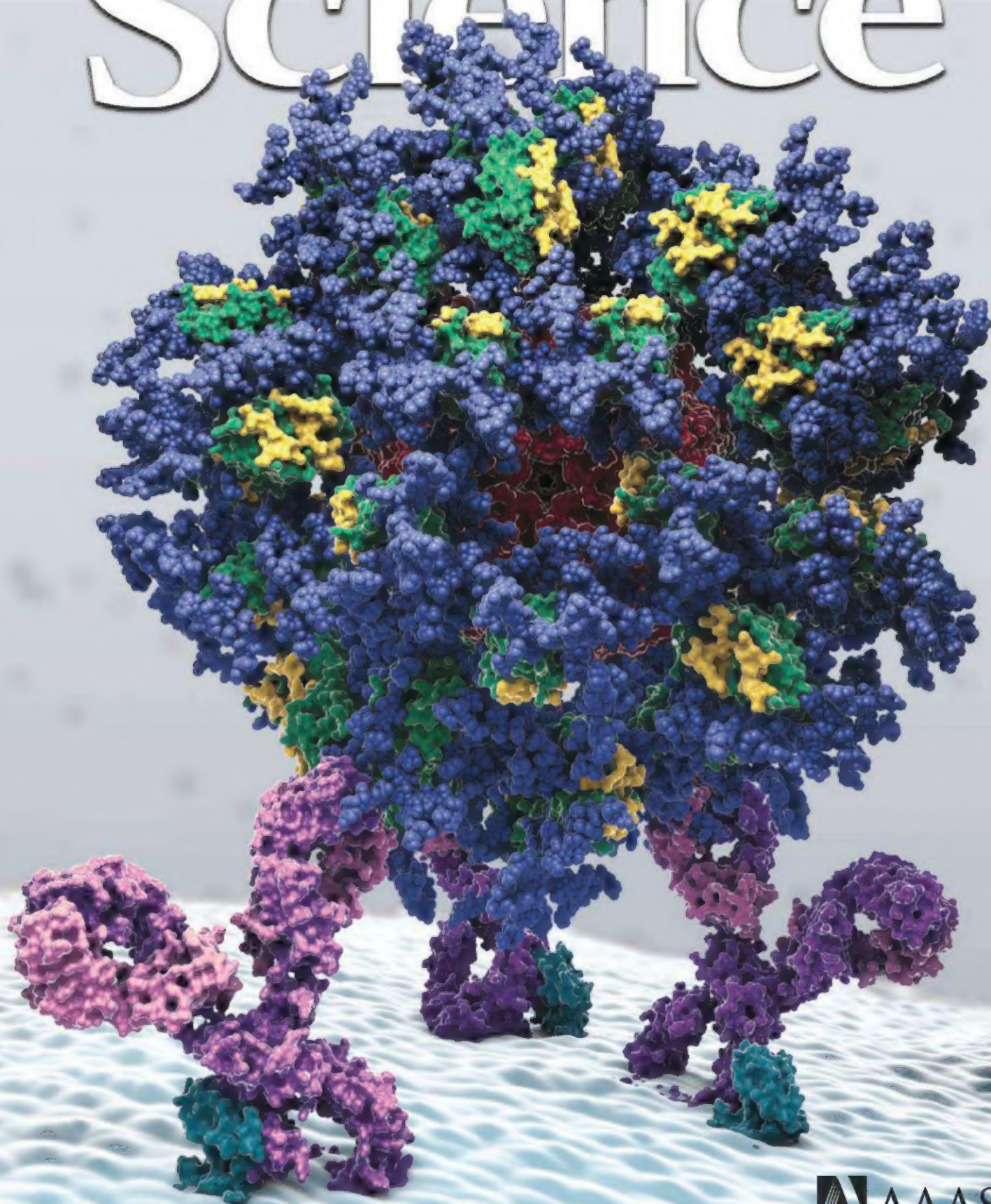
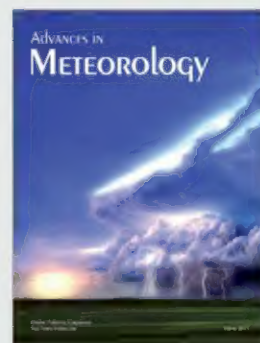
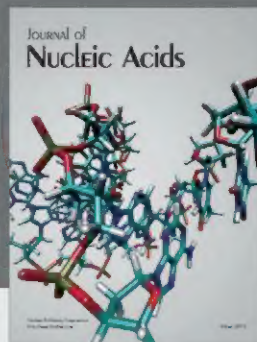
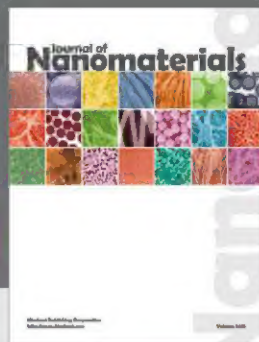
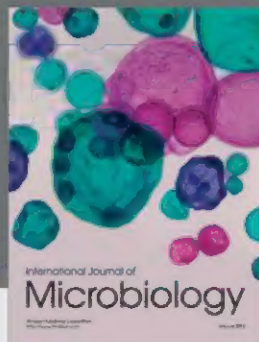


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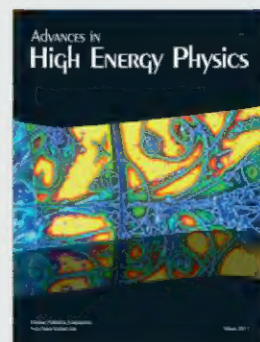
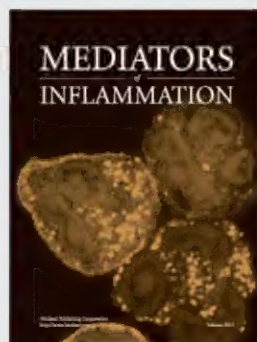
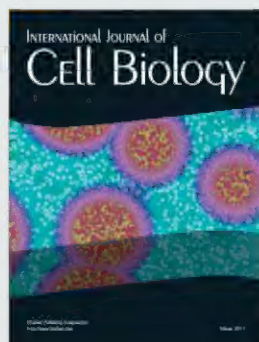
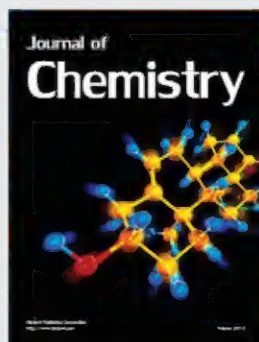
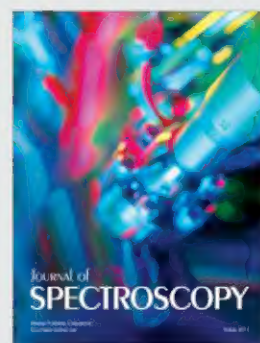
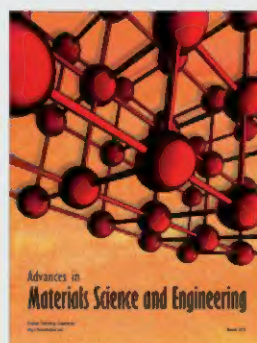
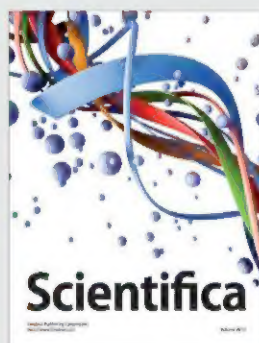
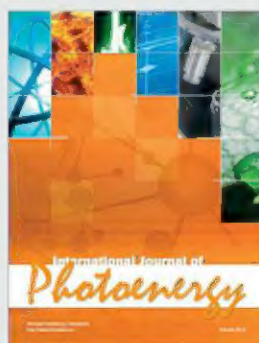
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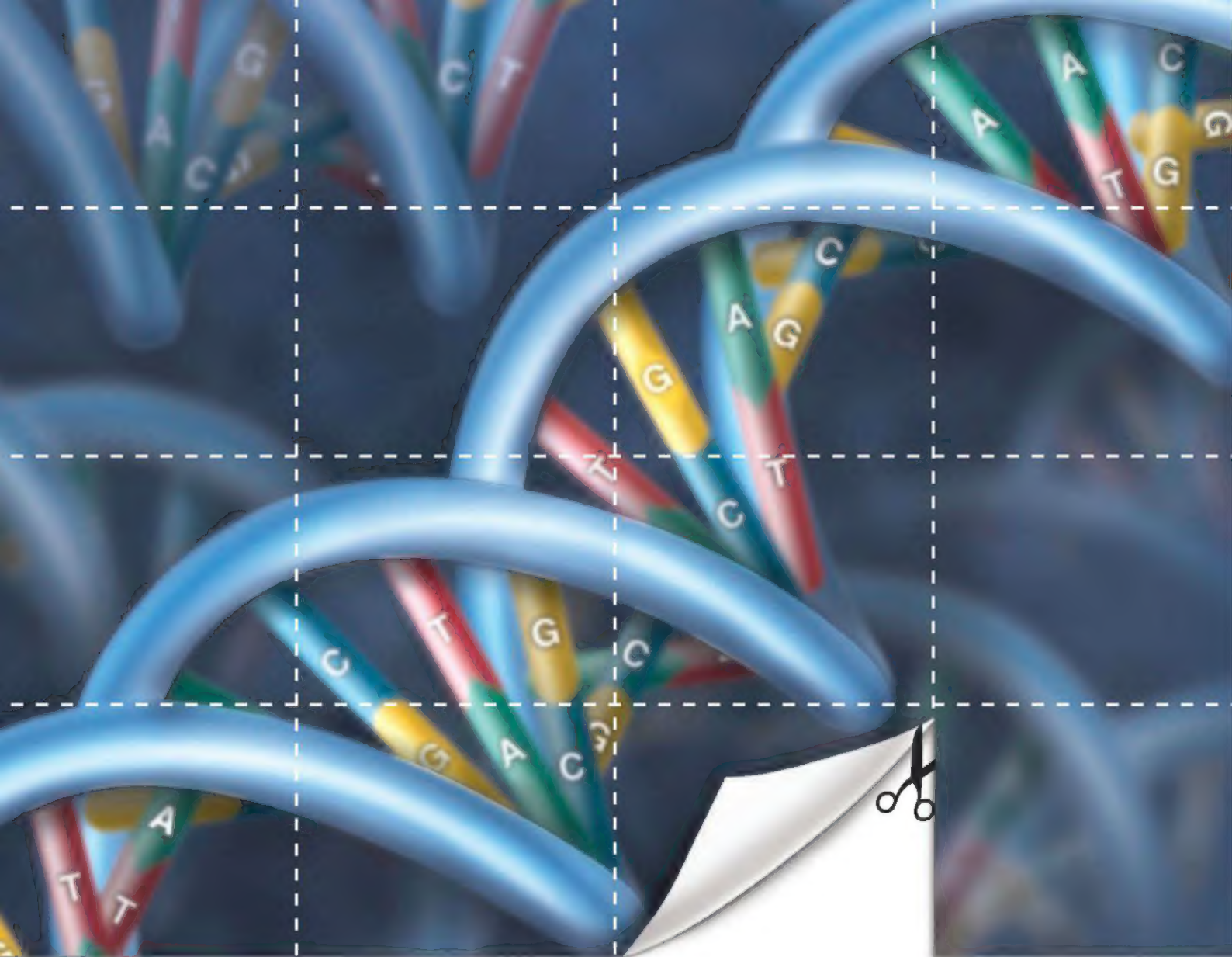
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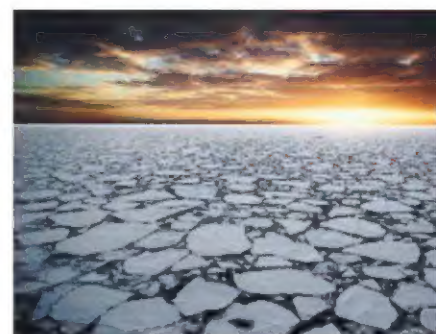
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COVER

Model of a candidate HIV vaccine prime immunogen (center) engaging germline B cell receptors (bottom) to initiate an antibody immune response. The immunogen is a virus-like nanoparticle, ~30 nanometers in diameter, displaying 60 copies of an HIV gp120 outer domain protein engineered to bind germline precursors of specific broadly neutralizing antibodies. This work has promising implications for HIV vaccine research. See page 711.

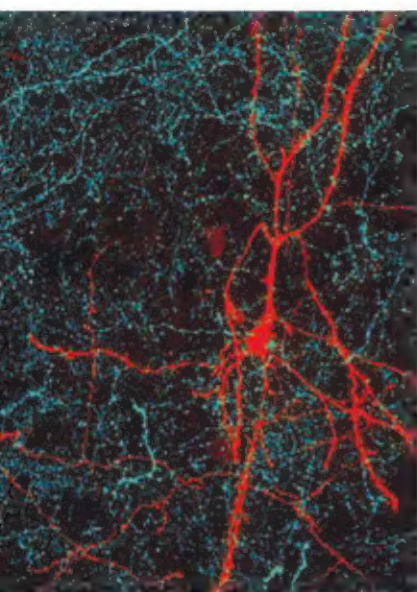
Image: Christina Corbaci, Adam Gardner, Joe Jardine, Sergey Menis, and William Schief, The Scripps Research Institute

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Simulating Foam Formation >>

Foams are easily made whether in the kitchen sink in the form of soap bubbles or a frothy head on the top of a quickly poured beer. Saye and Sethian (p. 720; see the Perspective by Weaire) describe the mathematical simulation of foam dynamics by decomposing the process into rearrangement, drainage, and rupture phases that are then linked by coupling the flux boundary conditions.



Defense and Counter-Defense

Provided a pathogen can enter the body and survive coughing and spluttering, peristalsis, and mucus, the first active responses the host evokes to an invading organism will be at the level of the first cell encountered, well before classical cellular immunity and antibody responses are initiated.

Randow et al. (p. 701) review the range of intracellular defenses against incoming pathogens and describe how compartmental boundaries within the cell provide multiple levels at which pathogens can be thwarted in their attempts to subjugate the cell to do their bidding. **Baxt et al.** (p. 697) review the range of evasion tactics that bacterial pathogens can summon to counter host repulsion and establish a niche in which to replicate and ensure onward transmission.

Building Better Vaccines

In the past few years, several highly potent, broadly neutralizing antibodies (bNAbs) specific for the gp120 envelope protein of HIV-1 have been discovered. The goal of this work is to use this information to inform the design of vaccines that are able to induce such antibodies (see the Perspective by **Crowe**). However, because of extensive somatic hypermutation, the epitope bound by these antibodies often does not bind to the germline sequence. **Jardine et al.** (p. 711, published online 28 March; see the cover) used computational analysis and in vitro screening to design an immunogen that could bind to VRC01-class bNAbs and to their germ-

line precursors. **Georgiev et al.** (p. 751) took advantage of the fact that only four sites on the HIV viral envelope protein seem to bind bNAbs, and sera that contain particular bNAbs show characteristic patterns of neutralization. An algorithm was developed that could successfully delineate the neutralization specificity of antibodies present in polyclonal sera from HIV-infected patients.

A Phase for Fano

In spectroscopy, samples placed between a steady light source and a detector are characterized based on the relative intensities of light absorbed at different frequencies. Temporal behavior—the relaxation of a photoexcited state—can be indirectly inferred from the absorption band shapes. The advent of ultrafast laser technology has enabled increasingly sophisticated measurements directly in the time domain. **Ott et al.** (p. 716; see the Perspective by **Lin and Chu**) present an analytical framework to account for asymmetric band shapes, termed Fano profiles, on the basis of a phase shift in the temporal dipole response.

Making Metamaterials

Controlling the propagation of electromagnetic waves is a key requirement in communication technologies. The components tend to be bulky, however, which can make it difficult to integrate with microelectronics circuits. Using arrays of metallic nanoantennae patterned on a substrate surface, **Shitrit et al.** (p. 724) fabricated a novel class of metamaterials: anisotropic materials

without inversion symmetry. The materials may pave the way to polarization-dependent nanophotonics.

Dust in the Clouds

Sulfate aerosols have the greatest radiative impact on climate systems. **Harris et al.** (p. 727) report that the oxidation of sulfur dioxide gas, catalyzed by natural transition metal ions mostly on the surface of coarse mineral dust, is the dominant pathway for sulfate production in clouds. In view of the growing sulfur dioxide emissions from large, industrializing countries, including this process in climate models should improve the agreement between models and observations.

Setting the Pace

The heart beats rhythmically throughout life. Highly specialized cardiac pacemaker cells control the timing of this beating. **Bressan et al.** (p. 744, published online 21 March) identified the embryonic location of the pacemaker precursors in early avian development and traced the cells throughout their incorporation into the heart. The events that establish the pacemaker lineage occur prior to the initiation of heart formation, and are governed, at least in part, by a class of Wnt signaling molecules.

Identical and Still Different

Even in monozygotic twins reared together, there are always observable differences reflecting the influence of individual responses. **Freund et al.** (p. 756; see the Perspective by **Bergmann and Frisé**) developed an inbred mouse model for studying the environmental influences on genetically identical animals and examined their effects on behavioral and neural development.



Infections Against Infection

In the same way that infection with the bacteria *Wolbachia* spp. can make *Aedes* mosquitoes resistant to dengue virus, there have been hints that these bacteria can interfere with the reproduction of malaria parasites. **Bian et al.** (p. 748) established a heritable *Wolbachia* infection in anopheline mosquitoes, which simultaneously suppressed the reproduction of malaria parasites within the adult female mosquitoes. The results hold promise for developing the model into a biocontrol agent to assist malaria control.

On Effective Leadership

MANY HAVE PONDERED WHY SOME NATIONS ARE MUCH MORE SUCCESSFUL THAN OTHERS. IS THE central factor an abundance of natural resources, wise traditional systems of governance, or the absence of serious conflicts? Or does a nation thrive merely as the result of a series of historical accidents? I believe that a primary determinant of success, often neglected, is how the leaders of its major institutions,* governmental and nongovernmental, are selected; how they in turn choose their deputies; and under what incentives they must operate.

The incentives part is perhaps the easiest to describe. To achieve anything of great importance takes time, but in an increasingly rapid-paced world of quarterly reports and sound bites, leaders often become hamstrung by short-term goals. And in large public organizations such as government agencies, leaders need to resist the inevitable pressures from employees to expand the responsibilities and budget of their particular division, even when other parts of the government (or society) are more qualified to meet a goal.

A core assumption here is that the leaders are qualified to lead. In a merit-based society, the selection of the most qualified person for each position of responsibility, independent of personal connections, background, sex, or age, has the obvious advantage of placing critical decision-making into capable hands. Much less obvious is the confounding fact that outstanding “A” individuals commonly have the self-confidence and discrimination needed to hire A, and whenever possible, A-plus, deputies below them. Conversely, when a poorly qualified person is selected as a leader, the opposite occurs. Feeling insecure, such B individuals will hire only B-minus or C deputies, giving rise to a propagating chain of mediocrity that degrades the entire institution.

When serving as president of the U.S. National Academy of Sciences (NAS), I befriended a very wise NAS Foreign Associate from Nigeria, Dr. Akin Mabogunje, who wrote an autobiography on the occasion of his 80th birthday.† In the final chapter, he describes the deplorable situation in his country created by “ascriptive rights”: rights that “just depend on being able to assert that one belongs to a group or part of the country to be able to lay claim to positions of authority or power.” As he starkly states: “The first consequence of putting unqualified individuals in important positions is their failure to grasp the nature of the opportunity being given to them to excel. Lacking the requisite mental capacity to cope with the challenges of the leadership position, they invariably turn their attention inward to weed out those whose presence reminds them of their own inadequacies. The mission of the institutions is thus lost in the pervasive fear of victimization for diligence. Any attempt at pursuing excellence at work is seen as a way of showing off the weakness of management and is punished rather than rewarded. In no time, the institution is manned by individuals who are willing to kowtow to the whims and caprices of the so-called managers or heads.”

I have seen the tragic consequences of the situation Mabogunje describes repeated over and over in nations around the world, from important U.S. institutions such as large school districts to international organizations associated with the United Nations. But I have also witnessed the opposite: the flourishing of institutions in which the deputies outshine the boss. This is why my advice to managers is to aim at hiring people who, at least in some respects, appear to be more talented than you are. This requires honest humility. But only in this way can a leader hope to achieve his or her goals.

It is only through a meritocracy in which leaders encourage creativity from outstanding subordinates and are primarily rewarded for long-term, rather than short-term achievements that a nation—and the world—can expect to meet the many challenges that lie before us.

— Bruce Alberts

10.1126/science.1239927

*B. Alberts, *Science* **338**, 443 (2012). †A. L. Mabogunje, *A Measure of Grace* (BookBuilders, Ibadan, Nigeria, 2011).



Bruce Alberts is Editor-in-Chief of *Science*.



BIOMEDICINE

The Benefits of Sequestration

About 170 million people worldwide are infected with hepatitis virus (HCV), which causes progressive liver disease, and current antiviral therapies are only partially effective and/or cause adverse side effects. HCV survival and replication require binding of the viral genomic RNA to a noncoding RNA of host origin that is abundant in the liver, called miR-122. Therapies that lower miR-122 levels have been tested in preclinical models of HCV infection with encouraging results.

Janssen *et al.* now report the results of a phase 2a clinical trial designed to assess the safety and efficacy of a chemically modified antisense oligonucleotide that sequesters miR-122 into a stable heteroduplex, thereby inhibiting its function. In a study of 27 HCV-infected patients receiving five weekly injections of the oligonucleotide, those receiving the highest dose showed a substantial decline in plasma HCV levels that persisted for about 15 weeks before rebounding. No significant side effects were observed and, as a bonus, a 25% reduction in plasma cholesterol levels was observed. — PAK
N. Engl. J. Med. **368**, 1685 (2013).

EDUCATION

Motivation + Skill = Success

Both motivational and cognitive variables play a role in academic achievement. The relationship between these variables and academic success at a specific time point has been described, yet there has been little research on whether these variables can predict long-term academic gains. Controlling for intelligence, Murayama *et al.* examined how motivation and strategies used by students to learn the material related to growth in academic achievement in mathematics over time. German students in grades 5 to 10 were assessed longitudinally for both math skills and self-reported motivation and learning strategies, and latent growth curve modeling was used to evaluate growth in mathematics achievement. Results showed that although the initial level of



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ANIMAL BEHAVIOR

A Social Shake-up by Song

Song, in birds, is a fundamental means of communication. Research over several decades has revealed how individuals learn, produce, and perceive song, but communication, by definition, occurs among multiple actors. Maguire *et al.* show that changes in song preference in individual female brown-headed cowbirds has cascading behavioral effects, altering social structure and interaction among all the birds within a flock. Targeted lesions that disrupted the song center of the brain in experimental females reduced their preference for the songs of dominant males. When these females were then released back into a mixed social group, their reduced song preference increased their solicitation of males other than their own mates. This in turn inspired other females to also increase solicitation and altered dominance structure among males, who now spent more of their time singing to females. Furthermore, network analysis showed that social networks that included females with lesions were less stable and connected than those containing control females. These results show that behaviors we often think of as specific to an individual, such as dominance, may in fact be emergent properties of group interactions. Simultaneously, they also emphasize the key role that individuals can play in shaping group dynamics. — SNV

PLoS One 10.1371/journal.pone.0063239 (2013).



achievement was strongly related to intelligence, motivation and learning strategies predicted growth in math achievement over time. Although correlational, these findings suggest that motivation and learning strategies should be further examined, as one of the ultimate goals in education is to enable sustainable learning. — MM
Child Dev. 10.1111/cdev.12036 (2012).

CHEMISTRY

Brushing Away Toxicity

Osmium tetroxide is among the most useful and versatile oxidizing agents of olefins, accounting for its widespread application in chemistry

research despite its dangerous combination of toxicity and volatility. Basavaraju *et al.* have constructed a microfluidic reactor that safely confines the compound without unduly compromising its reaction kinetics. The design incorporates high-surface-area nanobrush polymers that tether the osmium (Os) to the reactor while leaving it readily accessible to the incoming reagents. Coating conventional polydimethylsiloxane with a polyvinylsilazane layer before grafting these brushes rendered the interior of the reactor tolerant to organic solvents. Addition of tetrahydrofuran stabilized the

Continued on page 662



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catalytic quantities of the bound Os complex in the +6 oxidation state, after which olefins could be readily dihydroxylated or oxidatively cleaved through reaction with stoichiometric *N*-methylmorpholine-*N*-oxide or periodate, respectively. The system could be reused after 3 months with no degradation in performance. — JSY

Angew. Chem. Int. Ed. **52**, 10.1002/anie.201301124 (2013).

CHEMISTRY

Mimicking Rodlike Viruses

The self-assembly of one-dimensional (1D) structures that have a precise length can be particularly difficult because such assemblies are prone to aggregation, even though these structures are readily formed by rodlike viruses such as the tobacco mosaic virus. Ruff *et al.* have mimicked the assembly of 1D filamentous viruses by encapsulating double-stranded DNA—both linear strands and supercoiled circular plasmids—in water. They used a triblock structure to mimic the protein coat. A cationic spermine unit that binds to DNA is attached to a peptide that forms a compact coiled structure. The other end of the peptide bears a polyethylene glycol (PEG) tail that creates a hydrophilic exterior structure. These mushroom-shaped capsomers bind and encapsulate DNA (for DNA strands 1200 base pairs long, about 1.2 to 1.5 units bind per base pair) and can form structures up to 1.6 μm in length. The homogeneity of the linear structures

formed improved with longer PEG tails (5000 versus 2000 monomers), as determined by small-angle x-ray scattering and transmission electron microscopy studies. The larger structures formed by the longer PEG chains appear to stiffen the structure and allow the DNA to work effectively as a linear template, and decrease the fraction of shorter structures that form when DNA buckles and folds back on itself. — PDS

J. Am. Chem. Soc. **135**, 6211 (2013).

BIOMEDICINE

Peptide Prevention

One of the many challenges faced by cancer survivors is the possibility of relapse. Theories as to why cancers relapse after initially responding to therapy are varied and are likely to depend,

at least in part, on the type of treatment the patient receives. Engels *et al.* investigated why tumors relapse after successful adoptive T cell therapy in mice. Mice received an injection of a fibrosarcoma cell line that expressed one of a series of defined peptide antigens. Once tumors were established, they were then treated with T cells specific for that antigen. In vitro, T cells killed the tumor cells independent of the peptide's affinity for binding to major histocompatibility complex (MHC), which presents the peptide to T cells. In vivo, however, only mice that received tumors expressing peptides that were able to bind to MHC with high affinity remained cancer-free. Relapse was observed when affinities were less than 10 nM and was associated with less efficient cross-presentation of peptide antigens by stromal cells surrounding the tumor and reduced stromal cell death. Thus, strong interactions between peptide antigens and MHC may prevent relapse by promoting robust T cell responses that target the tumor itself and the surrounding tissue. — KLM

Cancer Cell **23**, 516 (2013).

BIOCHEMISTRY

Attacked by Radicals

Some bonds are easy to break, and enzymes can handle these via general acid-base catalysis; other bonds are tougher nuts to crack, hence enzymes call upon more potent chemical weaponry, such as unpaired electrons. Phosphonate

metabolism protein Phn] is a member of the family of radical *S*-adenosyl-L-methionine (AdoMet) enzymes and breaks the C-P bond in methyl phosphonate. Kamat *et al.* show that the redox active iron-sulfur cluster in Phn] converts

AdoMet to the Ado-CH₂[•] radical. They use isotopic labeling and mass spectrometry to establish that this radical pulls off the *pro-R* hydrogen from a nearby glycine residue to create a glycy radical, which turns around and pulls off the hydrogen from a nearby cysteine to create a thiyl radical. It is this third radical that attacks the methyl phosphonate substrate, breaking the C-P bond by homolysis to form a thiophosphate intermediate and freeing the methyl moiety to grab the *pro-S* hydrogen (from the same glycine) as it exits as methane. The thiophosphate collapses intramolecularly to a cyclic phosphate, regenerating the sulfhydryl side chain, which can then go on to catalyze additional rounds of C-P cleavage in concert with the glycy radical. — GJC

Nature **497**, 132 (2013).

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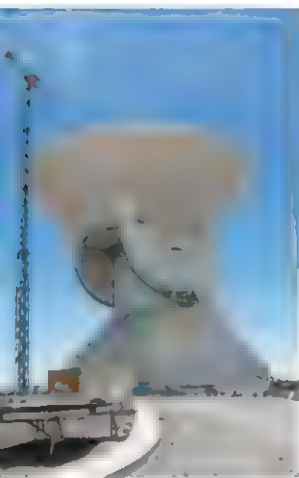
AROUND THE WORLD



Puebla, Mexico 1

Binational Telescope Begins Scientific Observations

The Large Millimeter Telescope (LMT) in Puebla, Mexico, will begin its first scientific observation season next week. Perched on the summit of a 4600-meter dormant volcano called Sierra Negra, the LMT will study regions of star, galaxy, and planet formation that cannot be explored by optical telescopes.



Looking up. The LMT starts its first season next week.

The LMT's 50-meter antenna makes it the largest single-dish, steerable millimeter-wavelength telescope in the world. Its wide field of view will complement the Atacama Large Millimeter/Submillimeter Array (ALMA), a suite of 66 antennas inaugurated in March in Chile's northern desert. "To put what ALMA is seeing in

context, it really helps to be able to image a much larger region in the same part of the sky" with the LMT, says Alison Peck, an astronomer at the National Radio Astronomy Observatory in Charlottesville, Virginia, who works on ALMA.

The LMT is the result of a binational collaboration between the University of Massachusetts, Amherst, in the United States and Mexico's National Institute of Astrophysics, Optics and Electronics, which provided about 70% of the telescope's approximately

\$180 million construction budget. The telescope's first observation season will last 10 weeks, with a second, longer season beginning in November. <http://scim.ag/MexLMT>

Amsterdam 2

Journals Adapt to U.S. Trade Sanctions on Iran

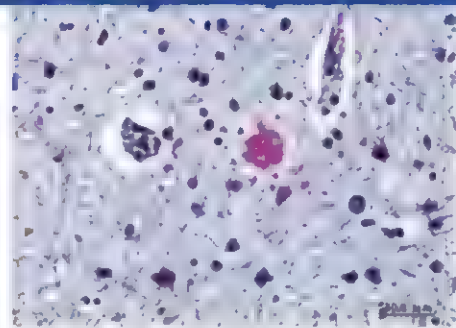
Scientific journals are being asked to help tighten U.S. trade sanctions on Iran. On 30 April, the publishing behemoth Elsevier, based in the Netherlands, sent a note to its editorial network saying that U.S. editors and reviewers must "avoid" handling manuscripts if they include an author employed by the government of Iran. Under a policy that went into effect in March, even companies like Elsevier that are not based in the United States must prevent their U.S. personnel from interacting with the Iranian government.

The sanctions, aimed at punishing Iran for its pursuit of nuclear technology, have been broadened from previous rules issued by the enforcement agency, the U.S. Office of Foreign Assets Control (OFAC), a division of the Treasury Department. According to a treasury official, OFAC still allows journals to publish articles authored by nongovernmental scientists from Iran and other sanctioned countries. But OFAC now insists that all U.S. citizens, no matter who employs them, comply with the sanctions. <http://scim.ag/JournalsIran>

Bethesda, Maryland 3

Seeking Sex Differences in Alzheimer's Disease

The Foundation for the National Institutes of Health this week announced a \$100,000 research challenge for proposals for research studies that reveal differences between males



Brain plan. A new challenge offers a prize for ideas to study gender differences in Alzheimer's.

and females in the development of Alzheimer's disease. Women are twice as likely as men to develop Alzheimer's disease—at least in part because they live longer, says Meryl Comer, president of the Geoffrey Beene Foundation Alzheimer's Initiative, which is co-sponsoring the challenge. But other differences may contribute to that discrepancy, and "it's time to find out," she says. "To me this is the biggest women's issue since breast cancer."

The challenge requires written proposals of hypotheses that include a "rationale supported by available data" and a detailed research plan. Half of the prize money will go to the winning hypothesis. The remaining \$50,000 will be divided among up to five finalists whose hypotheses mined existing databases for potential clues (a particular goal of the challenge, Comer says). To receive the money, the awardees must also grant the initiative a non-exclusive license to move forward with the proposed research plans.

NEWSMAKERS

Three Q's

As deputy director-general of the China Center for Disease Control and Prevention in Beijing, microbiologist **George F. Gao** is in the trenches for the H7N9 avian influenza outbreak, which has killed 27 in China since March. Gao heads up a team of researchers

NOTED

>A proposed Nikola Tesla museum got a big boost last week: Thanks to a crowdfunding campaign by Tesla enthusiasts Friends of Science East Inc.—with big help from online comic Matthew Inman of *The Oatmeal* (*Science*, 31 August 2012, p. 1024)—**the Friends have bought Wardencliff**, Tesla's Shoreham, New York, laboratory. Next up: fundraisers to restore the lab and build a learning center.

at the Chinese Academy of Sciences' Key Laboratory of Pathogenic Microbiology and Immunology who have been studying the virus, with the goal of understanding how it compares to H5N1.

Q: What are the challenges in controlling the virus's spread?

G.G.: Because H7N9 has a low pathogenicity in domestic poultry and migratory birds, it's hard to identify which animals are carrying the virus—and difficult to implement prevention and control measures. And its real threat [in humans] has yet to be evaluated.

Q: How will H7N9 affect other avian flu research?

G.G.: The global scientific community has been talking about the potential threat avian influenza viruses pose to humans for a while, but no one ever even thought about anything like H7N9. This is the first time the N9 subtype has been seen to infect humans. That should remind us that everything is possible, and we need to stay open-minded. And with animal surveillance, we need to cover all the subtypes.



Q: What should be done next?

G.G.: First, scientists need to do transmissibility studies in ferrets or monkeys to confirm whether there is sustained mammal-to-mammal transmission of H7N9. Then we need to answer the question of how the virus binds to the human receptor. My lab is working on that now.

Eugenie Scott to Step Down At Science Education Center

After 26 years, **Eugenie Scott** will let someone else shoulder the burden of defending evolution and climate change against the naysayers. The founding executive director and "public face" of the National Center for Science Education announced her retirement this week from a job that hasn't gotten any easier but perhaps has never been more important.

"She's incomparable, irreplaceable, and indispensable," says Kenneth Miller, a biology professor at Brown University and a key figure in one of the center's most decisive victories, a 2005 court case that blunted an



Defender. Eugenie Scott stands outside the federal court where the Pennsylvania evolution case was heard.

attack on evolution by the Dover, Pennsylvania, school district. He says that Scott was masterful at building the coalition needed to win the case.

Trained as a physical anthropologist, Scott joined the fledgling organization based in Oakland, California, in 1987 and built it into a 15-person, \$1.2 million-a-year operation. "I think all nonprofits hope someday to put themselves out of business," says Scott, now 67. "But these issues are like background radiation. Once you starting to look for the opponents, you'll find them."

THEY SAID IT

"The strength of ... *DSM* has been 'reliability'—each edition has ensured that clinicians use the same terms in the same ways. The weakness is its lack of validity."

—Thomas Insel, director of the National Institute of Mental Health, in a 29 April blog on the *Diagnostic and Statistical Manual of Mental Disorders (DSM)*. <http://scim.ag/NIMHDSM>

Scientists Win for Low-Cost Innovations

A pair of Rice University bioengineers who created a program in which students design low-cost health technologies for the developing world has earned the 2013 Lemelson-MIT Award for Global Innovation.

Rebecca Richards-Kortum and **Maria Oden** established Beyond Traditional Borders in 2006. Students work with clinical partners to develop technologies and >>



Jamestown Settlers Resorted to Cannibalism

When settlers in the Jamestown, Virginia, colony ran out of food in the winter of 1609 to 1610, they ate horses, dogs, rats, snakes, shoes—and, some contemporary records suggested, each other. On 1 May, scientists revealed the first physical evidence of that survival cannibalism at the colonial fort: deep cuts carved into bones that once belonged to "Jane," a 14-year-old English girl. Her damaged skull and fragments of her tibia were found among the butchered remains of horses and dogs dated to the period, known as the "starving time." The extensive chop marks on the bones and their location among other food waste led anthropologists to conclude that Jane's facial muscles, tongue, and brain were eaten by the starving colonists after she died. Only 60 out of 300 Jamestown settlers survived the starving time, but the colony persisted to become the first permanent English settlement in North America. <http://scim.ag/Jamescann>

>>NEWSMAKERS

then field-test their ideas during an 8-week internship in Latin America or Africa. So far, the program's more than 3000 students have developed 58 health technologies that are in clinical trials or already in use, including a respiratory system based on aquarium pumps to help premature babies breathe and a portable field microscope that uses a battery-operated, LED-based flashlight as a light source. Richards-Kortum and Oden plan to donate the \$100,000 prize money to renovate the neonatal ward of a partner hospital in Malawi.

FINDINGS

More Clues to Origins of Frog-Killing Fungus

Infections by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) have devastated amphibian populations around the world, causing the decline or extinction of 200 species. By sequencing the genomes of 29 *Bd* strains, Erica Bree Rosenblum from the University of California, Berkeley, and her colleagues discovered that many of the strains had arisen well before the epidemic started and differ significantly, they reported this week in the *Proceedings of the National Academy of Sciences*. "We've been treating



Fatal fungus. The genome of a frog killer reveals its complicated nature.

this pathogen as one singular thing, but it has different subgroups with different properties," Rosenblum says. Unfortunately, that will complicate efforts to pin down where, when, and how the killer fungus arose.

A second study, published this week in *PLOS ONE*, adds weight to a prevailing theory that humans helped to spread the fungus by importing and releasing the African clawed frog, used 60 years ago for human pregnancy testing. San Francisco State University ecologist Vance Vredenburg and his colleagues found *Bd* in clawed frog museum specimens from California and Africa, suggesting that imported infected frogs carried the fungus from Africa to California, where it spread to other species.

Slow Malaria: Infect Mosquitoes

Infecting a mosquito with a bacterium may not seem like a big scientific feat—but it is if the infection might just stop the insect from transmitting malaria.

A study by Xi Zhiyong of the Michigan

State University in East Lansing, published on page 748 of this issue, shows that it's possible to infect *Anopheles stephensi*, the main insect carrier of the malaria parasite in South Asia and the Middle East, with *Wolbachia*, a bacterium that employs shrewd genetic tricks to spread rapidly through insect populations. Motivated by studies showing that mosquitoes harboring the bacteria spread pathogens less efficiently, researchers had tried in vain for more than a decade to establish a *Wolbachia* infection in *Anopheles*.

Field studies with *Wolbachia*-infected *Aedes aegypti* mosquitoes, which can transmit dengue, have taken place in Australia (*Science*, 10 December 2010, p. 1460) and are under way in Vietnam, led by Scott O'Neill of Monash University in Australia. "I'm very jealous" of Xi's team, O'Neill says. Xi says field studies in India are the next step; so is trying to infect *A. gambiae*, the most important malaria vector in Africa, with *Wolbachia*. <http://scim.ag/mosqbact>

Science LIVE

Join us on Thursday, 16 May, at 3 p.m. EDT for a live chat on **bees and pesticides**. Are these important pollinators in danger? <http://scim.ag/science-live>

Random Sample

Alien? Nope, Tiny Skeleton Is Human

Ten years ago, a bizarre 6-inch-long skeleton was reportedly found in Chile's Atacama Desert. Rumors flew over what the skeleton was. (A mummified fetus? A deformed child?) Producers of a documentary called *Sirius*, which premiered last month, speculated that the mummy was evidence of alien life.

Now, a Stanford University scientist has put to rest doubts about which species Ata belongs to: ours. Last fall, immunologist Garry Nolan, director of Stanford's National Heart, Lung, and Blood Institute's Proteomics Center for Systems Immunology in California, heard about Ata from a friend and contacted the filmmakers, offering to give them a scientific readout. They asked him to give it a shot.

Nolan sought clues in Ata's genome. Given the dryness of the Atacama Desert, he initially presumed the specimen was tens or hundreds of thousands of years old. But "the DNA was modern, abundant, and



Otherworldly? X-rays show that Ata is no hoax. And its DNA shows it's no alien.

high quality," he says; Ata is probably a few decades old. And after mapping more than 500 million reads to a reference human genome, Nolan concluded that Ata "is human, there's no doubt about it." Moreover, the specimen's mitochondrial DNA reveals that its mother was from the west coast of South America: Chile, that is.

X-rays revealed that Ata's skeletal development seems equivalent to that of a 6- to 8-year-old child. Genetic and bone marrow tests may determine whether Ata had dwarfism, or suffered from progeria, a rare, rapid aging disease. Once the analyses are complete, Nolan says, he'll submit his findings for peer review.

Regardless, Ata is not an elaborate hoax, Nolan says: The x-rays show that these are real bones. "You just couldn't fake it," he says, adding, with a laugh, "unless you were an alien." <http://scim.ag/6inchskel>

AIDS RESEARCH

More Woes for Struggling HIV Vaccine Field

Devastating results from two trials have sent researchers scrambling—again—to reassess HIV vaccine strategies, and they have raised concerns about a vector widely used in vaccine and gene therapy studies. The double whammy suggests that a successful vaccine, the most effective way to slow if not end the epidemic, lies far in the future.

The first piece of bad news came on 22 April, when a board monitoring the largest HIV vaccine trial then under way recommended that investigators pull the plug. The U.S.-based study, which involved men and transgender women who have sex with men, tested a combination of two vaccines. One contained HIV genes stitched into bland bits of DNA, and the other used a weakened version of an adenovirus, which causes the common cold, to carry the genetic payload of the AIDS virus. The monitoring board, which got an early look at the data, learned that 41 vaccinated people had become infected with HIV compared with 30 in the placebo group. The National Institute of Allergy and Infectious Diseases (NIAID), which has invested \$77.2 million and 11 years in the study, promptly ended vaccinations in the trial known as HVTN 505.

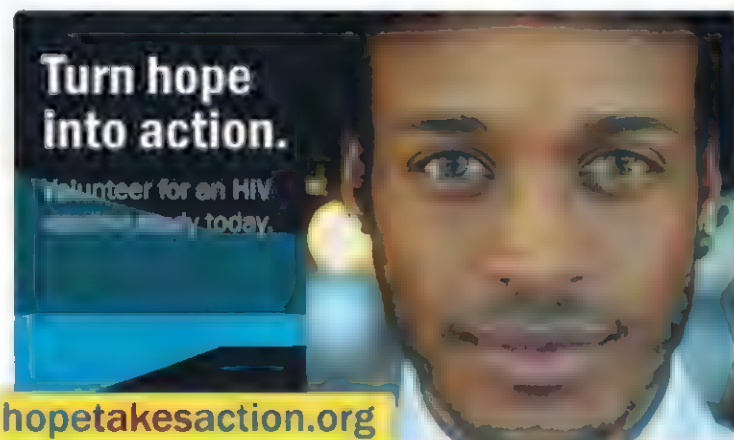
"This was a huge blow," says Scott Hammer, an infectious disease specialist at Columbia University Medical Center who co-chairs the study. "But it was a clear answer to the question: The vaccine absolutely did not work."

Although the number of additional infections in vaccinated people was not statistically significant, "even a few infections more in the vaccine group is worrisome and has to be looked into," Hammer says. "Was there something about this vaccine regimen that put people more at risk?"

The "something" now under the spotlight is the adenovirus vector. In 2007, an AIDS vaccine study called STEP that used the same "Ad5" vector was halted after it,

too, found more infections in vaccinees than placebo recipients (*Science*, 5 October 2007, p. 28). An analysis suggested that infections disproportionately occurred in uncircumcised vaccinees and those who had preexisting antibodies to Ad5, so HVTN 505 recruited only participants who did not have those supposed risk factors. HVTN 505's results "upset the hypothesis" that Ad5 makes only select people more susceptible to HIV, Hammer says.

Now, *Science* has learned that a recently completed follow-up analysis of a sister trial



Hope runs out. A large trial of an HIV vaccine ended early after an interim analysis found that it had no chance of proving its worth.

to STEP in South Africa, which also ended in 2007, has found a statistically significant link between the Ad5 vaccine and the risk of HIV infection. In all, the "Phambili study" found HIV infections in 37 placebo recipients versus 63 vaccinated heterosexual people—and most of those infections occurred years after the last vaccination. "I believe there is an increased rate of HIV infection in the vaccine arm, but the next question is what caused it?" says study chair Glenda Gray, a pediatrician at Chris Hani Baragwanath Hospital in Soweto. "It is difficult to explain biologically. We've been scratching our heads."

These results have far-reaching implications, as adenovirus vectors are widely used in experimental vaccines and gene therapy studies. "I think enough has happened now for us to be worried," says Dennis Burton,

an immunologist at the Scripps Research Institute in San Diego, California. "For the future, we've certainly got to pause on Ad5 and probably all adenovectors."

Aside from safety, the failure of the HVTN 505 trial underlines the staggering challenges that lie ahead for the HIV vaccine effort. "The simple things simply haven't worked," says Nobel laureate David Baltimore of the California Institute of Technology in Pasadena, who closely follows the field.

Many researchers contend that protecting people from HIV infection requires antibodies capable of "neutralizing" myriad variants of HIV in test-tube experiments. Although investigators have identified dozens of these "broadly neutralizing" antibodies, no vaccine has yet prodded the immune system to make them. "There's a remarkable collection of first-rate intellects now focused on seeing how far you can go with antibodies,"

Baltimore says.

Some evidence suggests that an antibody that merely binds to HIV but does not neutralize it can prevent infections. A vaccine trial in Thailand led by the U.S. Military HIV Research Program (MHRP) and completed in 2009 found 31% protection and linked its success to antibodies that bind to an HIV region called the V2 loop (*Science*, 2 October 2009, p. 26). But HVTN 505 also stimulated V2 binding antibodies, which clearly did not protect.

MHRP virologist Jerome

Kim, who headed the "RV144" Thai trial, notes that the quality and quantity of V2 antibodies differed between the studies—and he says that they likely are but one correlate of protection. "RV144's focus on V2 antibodies has created something to look at, but it isn't going to be the answer for all the studies," Kim says.

Coupled with the disappointment and confusion about the failure of HVTN 505 is the stark realization that the HIV vaccine development pipeline has little flowing through it. Other than a large-scale trial of a new and improved version of RV144 set to start in South Africa in 2015, all HIV trials currently in the works are at very early stages. Burton of Scripps says: "It's time for reflection, isn't it?"

—JON COHEN

NASA

Planetary Scientists Casting Doubt On Feasibility of Plan to Corral Asteroid

NASA's new plan to capture a tiny asteroid in deep space and lodge it in the Earth-moon system so that astronauts can get their hands on it is certainly audacious. But instead of cheering, a lot of planetary scientists are dubious about the mission's chances of success. They also say that the claimed side benefits, should the mission somehow succeed, are largely imaginary.

Most asteroid scientists were surprised to hear NASA announce last month that its 2014 budget request includes \$105 million to begin work on a mission that would send a robotic spacecraft to capture an asteroid as early as 2019 and haul it back so that astronauts could rendezvous with it by 2022. In addition to fulfilling President Barack Obama's promise to have astronauts explore an asteroid by 2025, NASA says that the mission will accelerate efforts to protect the planet from marauding asteroids and hone the skills and technology needed to tap their mineral resources.

But "there's a real credibility gap here," says Mark Sykes, chair of NASA's Small Bodies Assessment Group. "A small group at [NASA] headquarters with little consultation with subject matter experts thought [the retrieval mission] would be a great headline. But that's not enough."

The seed for the mission was a study conducted by the Keck Institute for Space Studies (KISS), a self-described "think and do tank" at the California Institute of Technology (Caltech) in Pasadena. The study drew on two workshops on asteroid mining held in 2011 and 2012 that involved 34 participants; only two of them specialized in small solar system bodies, one from Caltech and one from the nearby Jet Propulsion Laboratory (JPL), which Caltech runs for NASA.

In April 2010, Obama gave NASA the goal of sending astronauts to an asteroid—presumably in deep space—by 2025 as the next steppingstone on the way to Mars (*Science*, 18 February 2011, p. 841). But at the workshops, "we realized the only way to get a human mission to an asteroid by the mid-2020s was to move an asteroid closer to Earth," says Louis Friedman, executive director emeritus of The Planetary Society, a space advocacy group in Pasadena that he co-founded with Carl Sagan and Bruce Murray. Given its tight funding, NASA would not have the money to develop a spacecraft that could sustain astronauts for the 6-month round trip to an asteroid in deep space, Friedman says. Bringing the asteroid to the astronauts seemed like the only way to go.

The KISS study grew out of a seminar at

Caltech given by astrodynamist and independent entrepreneur Marco Tantardini, then an intern at The Planetary Society, on retrieving an asteroid to Earth's vicinity for mining. At the seminar, Tantardini and Friedman learned of a 2010 study by John Brophy of JPL that showed how solar-electric propulsion—so-called ion drive—could provide the energy needed to wrangle an asteroid to Earth.

"NASA jumped on it," Friedman says. The agency had already abandoned construction of a permanent moon base as the next steppingstone to Mars in favor of the easier and cheaper trip to a deep-space asteroid. In early January of this year, in response to the KISS report, the agency commissioned an asteroid retrieval feasibility study at JPL.

As in the KISS study, that analysis found that asteroid retrieval was likely doable if several challenges could be met. One logistical problem, says Brian Muirhead of JPL, who led the as-yet unreleased study, is coordinating an enhanced observing system so that all the instruments can be brought to bear before the newly discovered asteroid whizzes out of range. In addition, NASA will need to develop more powerful solar panels and an ion engine that can deliver the energy needed to divert a 7- to 10-meter, 500-tonne asteroid to the Earth-moon system. The whole mission "is kind of crazy, but that's what we're good at," Muirhead says.

"This proposal very neatly gives human exploration an objective within the budget projected for NASA," says space ana-

CREDITS (LEFT TO RIGHT): DARPA (2); NASA, NASA/ADVANCED CONCEPTS LAB

IDENTIFY



GOAL

By 2016, find a suitable asteroid for retrieval. It would be about 10 meters across, not too oblong, weigh about 500 tonnes, spin slowly, and come back in the early 2020s.

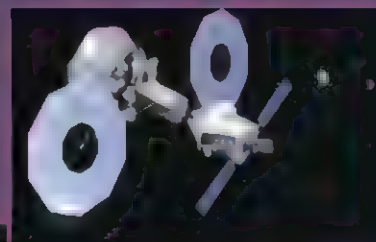
CHALLENGE

Don't blink. Astronomers searching for likely targets with ground-based telescopes will have to determine within days of discovery whether an asteroid meets the stringent criteria before it disappears again.

REDIRECT

GOAL

By 2017, design, construct, and launch a spacecraft capable of rendezvousing with an asteroid, taking it in tow, and hauling it back to the vicinity of the Earth-moon system.



lyst John Logsdon, a professor emeritus at George Washington University in Washington, D.C. "The bottom line is money."

However, asteroid specialists are more worried about the logistical and technical challenges to the schedule that NASA has been given. The KISS study found that "with the right ground-based observation campaign approximately five attractive targets per year could be discovered and adequately characterized." As it happens, astronomers coordinated by JPL actually spotted one candidate in late March, although it turned out to be on the small side for capture.

Still, scaling up that search so that it yields enough eligible candidates is going to be difficult, planetary scientists say. "Five a year to choose from is an outrageous number," says Alan Harris of La Cañada, California, who spent almost 25 years at JPL studying asteroids and how to find them.

The problem is twofold, scientists say. A suitable candidate must meet stringent criteria: It can't be too big, too massive, spinning too fast, or too oblong. In addition, its orbit must bring it back to the vicinity of Earth in the early 2020s in order to meet NASA's 2025 goal.

Then there's the brief window of opportunity available. All the characteristics of a suitable candidate must be determined in the few days or a week between when a passing tiny asteroid is discovered and when it moves beyond the range of the telescopes and radar needed for its characterization.

"That's a very difficult problem," says

Harold Reitsema of Boulder, Colorado. He is the lead designer for a privately funded, space-based mission to find larger, hazardous asteroids. Reasonable candidates would eventually be found, he says, but "NASA doesn't have that kind of time." NASA's working schedule has final target selection in 2016 in time for a 2017 launch of the capture spacecraft and a 2022 astronaut visit to the asteroid.

JPL's Muirhead says that he has "reasonable confidence we will find enough targets. We believe that with current [observ-

ing] assets and augmented assets becoming available, we will increase the rate from the current one per year to two per year, maybe five per year."

NASA's James Green, director of the Planetary Science Division, pleads with the community to "give us chance. There are plenty of things for the planetary community to be stressed about. This is not one of them." If Congress approves NASA's budget request, he says, scientists will know in a year or so just how efficient the search can be.

Asteroid scientists have a couple more beefs with NASA's plan. "Their claims about resource utilization and planetary defense are pretty empty," Reitsema says. The idea that studying a tiny asteroid up close would contribute to defending the planet from asteroid impacts "is just rubbish," he says. Ten-meter-wide asteroids simply can't penetrate the

atmosphere intact. At that size they disintegrate harmlessly, they don't impact.

Gaining insights into using asteroids as a resource—as a supply of water that could shield astronauts from radiation on long voyages, for example—would be "really tough," says JPL's Donald Yeomans, who contributed to the KISS study. For that you would need a water-rich carbonaceous chondrite asteroid, but "it's unlikely you'd get an object of that type," he says.

Asteroid scientists are also a bit miffed that NASA left them out of its planning. They had heard presentations on the concept, but "we just couldn't take it seriously," Sykes says. By early February, after realizing that NASA was indeed taking it seriously, he offered headquarters the services of its Small Bodies Assessment Group to help evaluate the idea. He got no response. NASA's Green says that "this is just the start. We will get them more involved."

Although it falls outside their expertise, asteroid scientists have one more complaint about NASA's latest plan. The whole point of astronauts going to an asteroid had been to gain experience for long-duration missions far from home, such as a trip to Mars. But "if you bring the asteroid to the astronauts instead of the other way around," Harris says, "you really aren't sending humans into deep space, or for that matter cutting any new ground over ... circling the Earth in the [International Space Station]." So other missions would be needed to gain the necessary deep-space expertise. —RICHARD A. KERR

Online

sciencemag.org

Podcast interview with author Richard A. Kerr (http://scim.ag/pod_6133).

CREDITS (LEFT TO RIGHT): NASA/ANALYTICAL MECHANICS ASSOCIATES, NASA

CHALLENGE

No precedent. The propulsion system would be the most powerful ion engine ever flown, and no one has ever captured



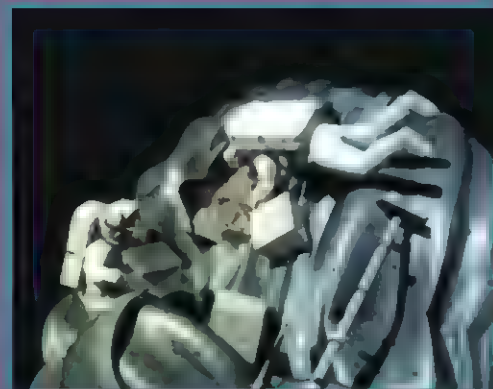
EXPLORE

GOAL

By as early as 2022, send astronauts to the asteroid. It would be safely stowed in orbit around the moon or between Earth and the moon, allowing astronauts to examine it and take samples over a few weeks.

CHALLENGE

Building it. NASA is well into development of the Orion Multi-Purpose Crew Vehicle and the Space Launch System to send it beyond low-Earth orbit, and Orion's first flight could come in 2014. But the trick is to keep the money flowing.



ASTEROID CAPTURE MISSION



U.S. SCIENCE POLICY

Proposed Change in Awarding Grants At NSF Spurs Partisan Sniping

The debate over a proposal from congressional Republicans that would alter the process of funding research at the National Science Foundation (NSF) heated up last week with an exchange of pointed public pronouncements.

Speaking at a major science policy conference, the president's science adviser and a leading Democrat heaped scorn on a bill authored by Representative Lamar Smith (R-TX), the chair of the House of Representatives science committee. In turn, Smith issued two statements that accused critics of distorting the legislation's intent for political purposes. In addition, a committee aide offered *Science* a detailed explanation of the draft legislation that seemed to confirm the suspicions of many scientists that the bill would allow Congress to intrude on the peer-review process. However, there are also hints that the two sides are looking for ways to defuse the controversy.

The battle was joined on 18 April when Smith circulated a three-page draft of his "High Quality Research Act" to committee members. It would require NSF to certify that any pending research grant addresses national interests and problems "of utmost importance to society." It also states that the research itself must be "ground breaking" and "not duplicative" of other federally funded research.

The draft appeared a month after Congress approved an amendment by Senator Tom Coburn (R-OK) to a 2013 spending bill that prohibits NSF from funding any political science studies unless the research advances economic development or national security. And it came 1 day after the science committee held two hearings in which Republi-

cans asked John Holdren, the president's science adviser, and acting NSF Director Cora Marrett to defend NSF's decision to fund several social science studies that members believed were of "questionable value." One week later, Smith wrote to Marrett asking for more information about five of the grants, including the comments of outside reviewers and the NSF program officer who decided to fund them.

On 2 May, Holdren, keynoting the annual S&T Forum sponsored by AAAS (which publishes *Science*), said that Smith's approach "makes no sense at all." He asserted that "imposing such a national interest criteria ... would throw out the basic research baby with the bath water insofar as basic research constitutes precisely that subset of research activity that is aimed at expanding knowledge without reference to possible applications."

The next day Representative Chaka Fattah (D-PA), the top Democrat on a House spending panel that oversees NSF, told the conference, "I don't think we should be fixing something that is not broken." Calling Smith's plan an attempt "to push aside or interfere with peer review at NSF," Fattah predicted that "there will be bipartisan opposition to it."

Within hours of Holdren's comments, Smith issued a statement explaining that the bill "maintains the current peer review process and improves on it by adding a layer of accountability" designed to "make sure hard-earned taxpayers' dollars are spent in ways that benefit the American people." But Smith clearly felt aggrieved; his statement noted that it was "disappointing that ... some have chosen to play politics and misrepresent the nature of the draft bill."

Not on the same page? White House science adviser John Holdren (right) has criticized a bill by Representative Lamar Smith.

The next day, a science committee aide was authorized to discuss the bill after *Science* acceded to his request for anonymity. The aide explained that the draft bill was prompted by "a certain number of NSF grants that have raised questions in the minds of several members of Congress about why these projects are being funded."

Legislators have noted that NSF has requested an 11% budget increase in 2014 and that the agency is funding only one in five of the roughly 40,000 research proposals it receives each year, the aide said. "So members are asking, 'If NSF has money to spend on these questionable grants, why are they asking for more money?' It goes to the amount of questionable awards versus productive awards. And that oversight is part of what Congress is supposed to do."

The bill draws a distinction between peer review and the final process of making an award, the aide said. "Congress is saying that we think an additional step is needed to solve the problem of why so many questionable grants are being awarded."

Despite the strong words on both sides, there may be room for compromise. A week after sharply criticizing Smith's letter to NSF as an unprecedented attack on peer review, the top Democrat on the science committee, Representative Eddie Bernice Johnson (D-TX), told *Science* that she "hopes to be able to work with Chairman Smith to identify a less destructive and more effective way to hold NSF accountable" to the Coburn amendment. And in his speech, Holdren said that defining national interest "as expanding the boundaries of knowledge would be fine by me. But that is not, I think, the intention of the members of Congress who have proposed this kind of language."

According to the science committee aide, however, Smith and Holdren aren't that far apart on defining national interest. The staffer noted that the bill's discussion of national interest ends with the words "by promoting the progress of science." The aide said that "if there's a better way to say it, I'd be happy to discuss it."

As for Smith's letter to NSF, the aide sought to allay the fears of many scientists by explaining, "We don't want the names of the reviewers. There's no intent to know who said what. We are simply asking NSF to tell us how it justifies the awards." NSF's response was due on 9 May.

—JEFFREY MERVIS

CREDIT: CHIP SOMODEVILLA/GETTY IMAGES

NEWSMAKER INTERVIEW: MICHAEL YAFFE

Boston Bombing Victims Aided by Biologist-Surgeon

It's not often that a systems biologist is also a trauma surgeon, active in the Army Reserve—and even rarer still that he treats the victims of a historic bombing. On 15 April, Michael B. Yaffe, who holds positions at the Massachusetts Institute of Technology (MIT) in Cambridge and Beth Israel Deaconess Medical Center in Boston, was rehabilitating a broken leg when two bombs exploded near the finish line of the Boston Marathon. Yaffe rushed to the medical center and was soon helping to treat victims. During the last day of the hunt for one of the bombers, he was locked down in the hospital; after injuries, both bombing suspects ended up there. (One died.) In an interview last week, Yaffe noted how injuries to humans parallel insults to cells, and he described the emotions of the “surreal” week, in which an MIT police officer was also killed, outside Yaffe's research lab, by the alleged bombers. “I have had a couple of bad dreams. But overall, I think I am doing pretty well with this. Let's see what happens over time,” says Yaffe, who is chief scientific editor of *Science Signaling* (published by AAAS, which also publishes *Science*). His remarks have been edited for brevity and clarity; a longer version of the interview can be found at <http://scim.ag/MBYaffe>.

—TRISHA GURA

Q: Can you talk about your experiences in the aftermath of the bombings?

M.Y.: I was in the gym. I saw on the television that there had been a bombing. I knew that my partner Carl Hauser was on for trauma at Beth Israel, so I sent him a text message. And it goes to show you how much I underestimated things, I said, “I doubt that it is a mass casualty. But just in case, let me know if you want me to come in.” Two minutes later, he sent me a text message that said, “Come on in.” They already had half a dozen people or so from the bombing in the emergency room. Everybody came together and worked in a way that I have never seen before. It functioned like a well-oiled machine.

Q: What was your role?

M.Y.: It was mostly as a surgical intensivist and a trauma surgeon. So most of what we did

was stabilize patients. Because the wounds were mostly lower extremity, it was really the orthopedic surgeons and plastic surgeons and vascular surgeons that did most of the operating. There were a total of 25 patients. I have to be careful that I don't violate HIPAA [patient privacy] rules. A number of them had traumatic amputations. Extremities missing. Many of them had combinations of injuries: broken bones, soft tissue injuries with significant tissue loss. Some burns. A lot of ... it was pretty horrendous.

Q: Did your Army Reserve background play a role?

M.Y.: There were a number of lessons that had been learned by the military in Iraq and Afghanistan that I think had a direct impact on the number of patients who survived this disaster. One was the early use of tourni-

M.Y.: My particular area of science is systems biology, which means you have to think in an integrative manner. When you take care of polytrauma patients, you also have to think very integratively, because it is not just a broken bone or a laceration. You have to think about more than the musculoskeletal system; you have to think about all the interactions between [body] systems. And you have to coordinate care among many different specialties, orthopedic, plastic surgery, vascular surgery, trauma surgery, pain management, infectious disease, social work, and psychiatry. So I think that sort of taking an integrative approach to biology lends itself naturally to taking an integrative approach to care.

The other part is that, as a PI [principal investigator], you have to learn how to manage a lab of people effectively because you have a goal and you have all these different assets. And you have to be able to use your assets most effectively to reach your scientific goal.

Q: How does the scientific amalgamation that has been your career translate to medicine?

M.Y.: Critical care medicine really is applied signal transduction. Sometimes you look at a patient and you say, “Well this is just a classic case of too much IL-1,” or “Wow, this is really what happens when you have a cytokine storm.” You can think through the physiology in molecular terms.

Also, we treat our patients in the intensive care unit, for example, by administering drugs that affect adrenergic receptors. It is all clinical pharmacology, which is really applied signal transduction.

Q: How do you deal with the fact that the suspects were at the hospital where you work?

M.Y.: I can't say whether I was involved in their care or not. But what I will say is that I think the attitude of most of us at the medical center is that we are here to take care of people. Same thing like in the Army. We take care of our soldiers and we take care of insurgents. Our role is to heal the wounded. We're not judges. That is a different part of our society.



Locked down. Michael Yaffe in his Boston hospital during the week he treated bombing victims.

quets. The other was rapid evacuation without necessarily trying to stabilize people. A number of the hospital personnel and I wrote peoples' injuries in indelible marker on their chests so that if the chart got separated from the patient, we could figure out which patient is which.

Q: After the first shootout, the hospital was in lockdown. What was that like?

M.Y.: It was surreal. I think we would have felt worse if we had been trapped at home and unable to get to the hospital. But it was an odd feeling to know that you could not leave.

Q: Is there some way that your scientific background played a role in your experience or management of events?

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Name change? This virus, infecting a cell, may soon be called Middle East respiratory virus coronavirus.

INFECTIOUS DISEASE

Amid Heightened Concerns, New Name for Novel Coronavirus Emerges

Much remains unclear about the deadly new coronavirus that surfaced in the Arabian Peninsula last summer and continues to kill people. But if an international group of scientists and public health experts gets its way, at least one thing about the pathogen will be clear soon: its name. In hopes of putting to rest confusion over what the virus should be called, the Coronavirus Study Group of the International Committee on Taxonomy of Viruses (ICTV) has proposed naming it after the Middle East—a recommendation supported by the World Health Organization (WHO) that could nonetheless prove controversial because some see geographical virus names as stigmatizing.

Under the proposal, the new virus would be called Middle East respiratory syndrome coronavirus (MERS-CoV); patients would likely be called MERS cases, and a global outbreak, if it erupts, might be the MERS pandemic.

The proposal, soon to be published as a letter in a scientific journal, comes just as Saudi Arabia has revealed a new wave of infections with the virus after a 6-week lull. As *Science* went to press, the country had reported 13 new cases and seven deaths in just 5 days—bringing the total worldwide to 30 cases and 18 fatalities.

The onslaught of cases, and the lack of information about what's happening on the ground in Saudi Arabia, has sparked fresh worries that the virus might start spreading between humans and trigger a pandemic. And

some scientists say they hope the new name will catch on soon because attention should be on the worrisome epidemiological situation. MERS-Cov “is a fine compromise and I compliment the study group for coming up with it,” says public health expert Michael Osterholm, of the University of Minnesota, Twin Cities. “But we have much more important work to do than argue about the name.”

Naming pathogens has become a tricky issue. Historically, many infectious disease agents—or the diseases themselves—have been named after the place where they were first found. But scientists and public health officials have increasingly shied away from that system to avoid stigmatizing a country, region, or town. The name for severe acute respiratory syndrome, a disease caused by another coronavirus, was carefully chosen in 2003 to avoid blaming China, where the pathogen emerged (*Science*, 15 March, p. 1264).

In the current case, confusion has reigned since the novel coronavirus was first reported by Ali Mohamed Zaki, an Egyptian microbiologist who isolated it in June 2012 from a patient at a hospital in Jeddah, Saudi Arabia, where Zaki worked at the time. Zaki sent the virus to Ron Fouchier's virology group at Erasmus MC in Rotterdam, the Netherlands, which characterized it further. The group provisionally called the virus HCoV-EMC/2012, short for human coronavirus-Erasmus MC.

That reference didn't sit well with Saudi health officials, who said that Zaki lacked

authorization to send the virus to Rotterdam in the first place. Still, most researchers have used HCoV-EMC so far. WHO had adopted the more neutral “novel coronavirus”—abbreviated either as NCoV or nCoV—a name that by its very nature was not meant to last.

Given the risks of the new virus, it is important to end the confusion, says Raoul de Groot, a veterinary virologist at Utrecht University in the Netherlands who chairs the Coronavirus Study Group. A name referring to Jeddah or Saudi Arabia would have been unacceptable, he says—let alone one including the word Arab or Arabian. Although MERS is easy to remember and pronounce, it remains a geographical name, and tweets calling it as an “insult” and an “ethnic slur” started flying after *Science* revealed the proposal in a story online. But the name does not have to be stigmatizing “because the Middle East is a very large region,” De Groot says. Infections with the new virus have so far been found in residents of Saudi Arabia, Qatar, Jordan, and the United Arab Emirates. There has also been a cluster of three patients in the United Kingdom, the first one of which is believed to have contracted the virus during a trip to Saudi Arabia.

Coming up with the name was “an incredibly difficult task,” De Groot says; to assure wide adoption of the name, the group consulted extensively with coronavirus researchers and other parties, such as WHO and the Saudi Ministry of Health; all eventually agreed, he says, and the letter will be signed not just by the nine study group members but a broader group. A WHO spokesperson confirms that the agency is on board and that the statement will be signed by at least one WHO official.

Meanwhile, just what the virus is up to is anyone's guess. The 13 recently reported patients have all been linked to a hospital in Hofuf, in Saudi Arabia's Eastern Province; but how they became infected, or whether there is human-to-human transmission, remains unclear. “It seems like a big cluster,” says Matthew Frieman, a coronavirus researcher at the University of Maryland School of Medicine in Baltimore, “but we still don't have much information at all about the patients and their co-morbidities,” or existing illnesses. Osterholm worries there may be more cases that Saudi Arabia hasn't identified or revealed yet. The country has to aggressively investigate the outbreak and promptly share the results with the world, he says. “It could make the difference between a local event and a global catastrophe.”

—MARTIN ENSERINK



Pesticides Under Fire For Risks to Pollinators

As the European Union moves to ban a popular type of pesticide, researchers struggle to assess exactly how dangerous the chemicals are to honey bees and other pollinators

IN APRIL 2008, SOMETHING WENT TERRIBLY wrong with beehives in southern Bavaria and the Upper Rhine Valley of Germany. Confused honey bees huddled trembling outside their hives rather than taking off for their morning flights. Many dropped dead. Over the next 2 weeks, the bodies piled up and more than 11,500 colonies were hit by the mysterious affliction. "It was a catastrophe," recalls Peter Rosenkranz, who studies bee health at the University of Hohenheim in Stuttgart.

Researchers quickly discovered the culprit: dust from seed corn that had been treated with a pesticide called clothianidin, one of the most toxic agrochemicals for honey bees. A seed company had neglected to use an adhesive to bind the pesticide to the seeds, so the farmers' planting equipment had been spewing dust that was extremely toxic to bees. Even though it was a rare mishap, the German government banned companies from

coating corn and certain other seeds with clothianidin and two related pesticides.

The event brightened the spotlight on the potential dangers of a family of pesticides called neonicotinoids. In 1999, France had banned one neonicotinoid, barring the use of imidacloprid on sunflower seeds after beekeepers suspected that it had killed a third of their colonies. Now, citing evidence from laboratory and field studies that neonicotinoids threaten honey bees and other pollinators, the European Union is poised to ban the use of three of the most common neonicotinoids in several crops by the end of the year.

Clearing the air. Planting pesticide-coated seeds can release toxic dust that kills bees. A collaboration is working to reduce the amount released.



Tagged. Radio chips have helped reveal how many bees lose their way after pesticide exposure.

In the United States, beekeepers and environmental advocates are demanding that the government suspend its approval of two of these pesticides, and recently they sued the Environmental Protection Agency (EPA), alleging that the agency hasn't adequately evaluated the chemicals.

Even as European regulators act, however, scientists are divided on whether pollinators are exposed to enough of the pesticides to pose a grave threat to their colonies, in part because of a paucity of data and the challenges of doing rigorous field trials. Pesticide and seed companies maintain that their products are safe when used properly. And many scientists remain unconvinced that chronic exposure to doses typically encountered in farm fields is a leading cause of pollinator declines. "The jury is still out," says Marla Spivak of the University of Minnesota, Twin Cities.

There's also debate about just how vital the seed treatments are for agriculture. The pesticide industry has warned that restricting neonicotinoids will jeopardize food security and farm incomes. Some researchers, however, are underwhelmed by that claim (see sidebar, p. 675) and note that some data suggest that neonicotinoid use may offer little boost to yields.

Dust-up

Developed in the 1980s, neonicotinoids are a major new class of insecticides. The compounds mimic the toxic effects of nicotine, which tobacco plants use as a natural insecticide. Neonicotinoids block receptors for the neurotransmitter acetylcholine; high doses cause paralysis and death in insects. Because neonicotinoids bind more tightly to the insect version of the receptor, low doses are relatively safe for other animals, including mammals.

EPA approved the use of a neonicotinoid called imidacloprid in 1994, and it has since become the most widespread insecticide in the world. Approved for use in about 140 crops and numerous garden and horticultural products, sales topped \$1 billion in 2009. Clothianidin and several other neonicotinoids have been commercialized over the past decade.

The most common use in agriculture is to coat seeds to protect them from soil pests. As the seed grows, it readily incorporates the compounds so that tender young plants are guarded as well.

CREDITS (TOP TO BOTTOM): JUERGEN TAUTZ/BEEGROUP BIOZENTRUM UN-VERSITAET WUERZBURG/HOBOS-TEAM, JIM PEARSON/AP

That means less pesticide is applied than if it was sprayed onto the plants. "It's a much more environmentally friendly way to apply a chemical," says David Fischer, director of environmental toxicology and risk assessment at Bayer CropScience in Research Triangle Park, North Carolina, a major manufacturer of neonicotinoids.

As use of neonicotinoids has grown, however, researchers have become concerned about their potential to harm birds, earthworms, aquatic insects, and especially bees. They have found traces of clothianidin and other seed-based pesticides in a large fraction of samples of dead honey bees from commercial beekeeping operations. "That's pretty astonishing" and "suggestive that the pesticides are related to the deaths," says Reed Johnson, an entomologist at Ohio State University's Agricultural Research and Development Center, Wooster. Honey bees and other pollinators can pick up the chemicals by feeding on nectar and pollen, or sipping on drops of liquid, called guttation, exuded by corn and other plants. The compounds are eventually fed to young bees back at the hive.

There's no debate that high doses of neonicotinoids kill pollinators, and studies suggest that chronic or intermittent exposure to low doses can also cause trouble. Over the past 5 years, for example, a host of findings have indicated that low doses can trigger behavioral effects in honey bees, such as memory and learning, which could affect foraging. The big question facing researchers is how to extrapolate from lab studies on individual bees to evaluate the impact on entire colonies, which are quite resilient. "You can lose a lot of bees and the colony is able to maintain itself," says Dennis vanEngelsdorp of the University of Maryland, College Park.

To study colony impacts, researchers have fed neonicotinoids to bees in colonies. But determining realistic doses experienced by bees is a sticky problem. Scientists don't know how much soil residue levels rise as fields are repeatedly planted with treated seeds. And homeowners can apply the pesticides at rates up to 120 times higher than farmers. "The actual exposure is likely higher than we think," Spivak says. New data could come

soon: The United Kingdom's Department for Environment, Food & Rural Affairs (DEFRA) has funded David Goulson of the University of Stirling to measure pesticide concentrations more widely in the landscape, including soils, crops, flowers, and hedgerows.

Smaller hives

Some scientists have started to focus on bumblebees, suspecting that they may be more vulnerable than honey bees because their colonies are much smaller. "You can have quite a dramatic effect compared to honey bee colonies," Rosenkranz says. In a high-profile study, Goulson and colleagues fed bumblebees pollen and sugar water containing imidacloprid. After the bees foraged in the open for 6 weeks, the team found 85% fewer new queens in the colonies that had been exposed to the pesticide, they reported in *Science* (20 April 2012, p. 351). "To me, the evidence is pretty close to overwhelming" that exposure has big impacts, Goulson says.

Scientists with DEFRA, however, objected. Goulson's doses were unrealistically high and thus "biased towards showing a deleterious

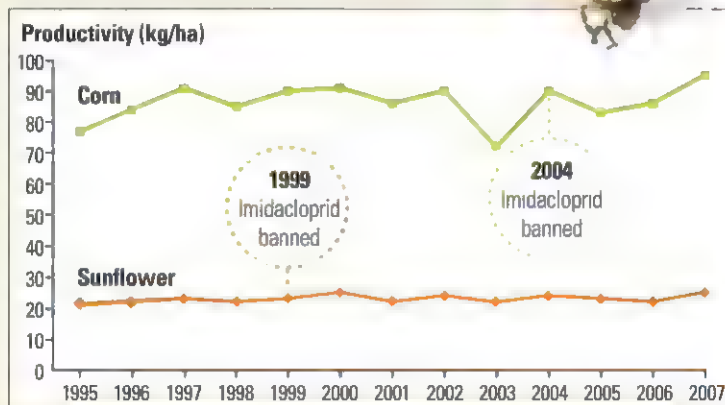
How Big a Role Should Neonicotinoids Play in Food Security?

Proponents of neonicotinoid-treated seeds claim that the chemicals offer many benefits besides killing pests, including improved plant vigor and higher yields. The business itself has certainly boomed. Almost all the corn and about one-half of the soybeans in the United States are grown from insecticide-treated seeds. "The companies are marketing them aggressively," says Paul Mitchell, an agricultural economist who studies pest management at the University of Wisconsin, Madison.

But how important are neonicotinoid seed treatments for agriculture? Agronomist Palle Pedersen, technology manager for seed care at Syngenta, says that treated corn seed produces an extra 9 bushels an acre above a national average of about 160. "We've seen a dramatic yield increase," he says. But researchers studying soybeans and other major crops have found treated seeds can come up short.

A 2-year trial of treated soybeans in South Dakota, for example, found no yield benefit. Insecticide concentration in the plants was too low by the time the major pest, aphids, arrived, according to a study published last year in the *Journal of Pest Science* by Jonathan Lundgren of the U.S. Department of Agriculture in Brookings, South Dakota. He says that his findings mirror those of other trials. A worrying postscript: The neonicotinoids also harmed predators of the aphids, such as omnivorous pirate bugs (which feed on the soybean plant itself as well as aphids). Pedersen isn't convinced. "It's such a small data set, we can't draw a conclusion out of that."

Companies say that they have copious data to prove the efficacy of treated seeds. "Admittedly, they do not increase yield all of the time, but the larger body of data says that they do provide an increase in yield a high percentage of time," says William Hairston, director of product development for seed growth at Bayer CropScience in Research Triangle Park, North Carolina. Few of these data are peer-reviewed, however, and some scientists are skeptical, saying that the trials often combine insecti-



Steady. Farmers kept yields after France banned neonicotinoid-treated seeds.

cide with fungicides, which are known to help prevent losses from disease.

Another reason that some scientists debate the overall value of the seed treatments is that the pests they target—such as wireworms, Japanese beetles, and seed corn maggots—are rarely major problems, or are already resisted by genetically modified crops. Still, with sky-high commodity prices, farmers don't want to risk lower yields, and want to guard against any potential pests. "The price of corn is so high, it's peace of mind," says entomologist Reed Johnson of Ohio State University's Agricultural Research and Development Center, Wooster.

Entomologist Christian Krupke of Purdue University in West Lafayette, Indiana, says that neonicotinoids are good tools, but overused. "They do not need to be on virtually every annual crop seed, every year," he says. "Our pest pressures do not justify the practice in fields that I and others have examined."

—E. S.



Diverse. Neonicotinoid-treated seeds are used in many crops, including soybeans, cotton, rice, and peanuts. Color serves as a warning.

effect,” they wrote in a review paper posted on the DEFRA Web site in March. Helen Thompson of DEFRA and others tried to resolve the question with a field study of 20 bumblebee colonies, some placed in fields of canola planted from treated seeds and others in fields of untreated canola. Unfortunately, the control failed; the bumblebees put into untreated canola had collected just as much clothianidin or imidacloprid in their nests, probably by flying to other fields. Nevertheless, DEFRA concluded in March that the neonicotinoids did not have any major effect on the hives. To James Cresswell of the University of Exeter, the bottom line is that it’s difficult to conduct a rigorous field trial.

Still, others are trying. One of the largest field studies, a \$950,000 effort funded by Bayer CropScience, started last summer in Ontario. A team led by Cynthia Scott-Dupree of the University of Guelph and Chris Cutler of Dalhousie University placed 20 colonies of honey bees in fields of blooming canola that had been planted with seed treated with the maximum approved dosage of clothianidin. Another 20 colonies were put in fields without treated seed, at least 10 km away. “This was our most complicated bee study to date,” says Fischer, adding that, so far, there are no significant differences between the colonies.

Complex mixtures

These results are unlikely to exonerate neonicotinoids once and for all, others say. One limitation is that the farms of southern Ontario are smaller and more diverse than in places such as the midwestern United States, where bees have fewer alternatives to neonicotinoid-treated crops. Another complication is that the effect of neonicotinoids may be exacerbated by additional threats. For example, Jeffery Pettis of the U.S. Department of Agriculture (USDA) Bee Research Laboratory and others have recently shown that neonicotinoids increase the risk that honey bees will contract viruses and a deadly microbial disease.

In addition, honey bees are exposed to many other chemicals. In a report released last week, EPA and USDA noted that insecticides called pyrethroids may pose a threefold greater hazard than systemic neonicotinoids. And those aren’t all. James Frazier of Pennsylvania State University, University Park, has studied commercial colonies that are trucked around the country to pollinate various crops. He found an average of more than six pesticides in pollen samples from the hives, with some containing as many as 30 chemicals. The situation may worsen over time because

honey bee combs accumulate pesticides, which, according to a 2011 study, can hinder larval development. The highest concentrations were of pesticides called acaricides that beekeepers use to fight a parasitic mite called *Varroa destructor*, which scientists say is the biggest threat to honey bee colonies. The dilemma is that without acaricides, mites will usually destroy colonies.

Not much is known about the impact of these pesticide mixtures, but they appear to spell trouble. A study online in *Nature* in October found a cumulative impact from a neonicotinoid and a pyrethroid. Ohio State’s Johnson and colleagues reported in *PLOS ONE* in January that certain acaricides and fungicides were more toxic to adult worker bees in combination, probably because fungicides inhibit the detoxifying enzyme cytochrome P450.

Another factor that may worsen the impact of pesticides is the way that people manage commercial beehives. Beekeepers often feed their insects high-fructose corn syrup to boost their activity. But that may hinder their ability to cope with pesticides, May Berenbaum of the University of Illinois, Urbana-Champaign, and colleagues reported last month in the *Proceedings of the National Academy of Sciences*. She found that honey (but not sucrose or corn syrup) contains naturally occurring compounds that up-regulate genes for detoxification. Adding one such compound, *p*-coumaric acid, to the sugar water fed to bees increased the bee’s breakdown of a common acaricide by about 60%.

One of the biggest unknowns facing neonicotinoid researchers is the impact on solitary bees, hiveless insects which are the least well-known kind of bee. North America has some 4000 species, which typically nest in the ground and are therefore likely exposed to pesticide residues in soil as well as in nectar and pollen. The bees have short lives and many are impossible to breed in the lab. “It is incredibly difficult to do research on solitary bees,” Rosenkranz says.

Europe acts

Despite such uncertainties, Europe is moving quickly to further tighten its regulations.

After the European Food Safety Authority (EFSA) released a report in January concluding that neonicotinoids posed “high acute risks” to pollinators, some governments proposed a partial ban in the European Union. The bid failed, however, in a vote of member nations. But as a result of parliamentary procedures, the European Commission was free

to act on its own. It decreed that, starting in December, farmers won’t be able to plant seeds treated with clothianidin, imidacloprid, or thiamethoxam, nor spray the chemicals on crops preferred by bees. There will be exceptions for use in greenhouses and on fields after flowering.

The EFSA review underpinning that decision was “hurried, incomplete and failed to take into account years of field monitoring, mitigation efforts, real-life applications and sound scientific studies,” complained Syngenta, which makes thiamethoxam, in a statement.

In the United States, EPA is slowly changing its process for evaluating neonicotinoids based on recommendations from a scientific advisory panel. The agency says that it is asking companies for new field studies and toxicity tests of honey bee larvae; some studies must now include measurements of residues over several years. But the pace is measured. “While EPA’s risk assessment framework for pollinators provides a roadmap that will eventually result in agency-approved guideline studies for pollinators, the work is very challenging,” the agency said in a statement to *Science*. Another reason for patience: EPA won’t complete its regular review of all the neonicotinoids until 2019.

In the meantime, industry scientists and academics are working to reduce the amount of neonicotinoid dust released by planting coated seeds. Bayer CropScience has commissioned researchers at three universities to test a seed mixture with a better lubricant that appears to reduce planting dust by 90%, says William Hairston, a director of product development in Research Triangle Park. The new formulation should be available to farmers next year.

And researchers continue to try to understand how the increasingly widespread chemicals are affecting ecosystems. “It’s been likened to living in a house with asbestos or drinking water from lead pipes” says Christian Krupke of Purdue University in West Lafayette, Indiana. Neonicotinoids, he says, “deserve the scrutiny they’re getting.”

—ERIK STOKSTAD

CREDIT: COURTESY OF SYNGENTA



China Heads Off Deadly Blood Disorder

To combat the spread of thalassemia, a Chinese province screens millions

NANNING, CHINA—The boy calmly sits on his hospital bed, slurping down a bowl of noodles, as new blood courses into his veins. He is by all appearances a normal 7-year-old, more interested in the cartoon blaring on TV than in his food. But without monthly transfusions and other treatments, the boy's life would be vastly different. Iron would build up in his blood. "The face changes color, and the liver and spleen become enlarged," says Chen Ping, a hematologist here at Guangxi Medical University. "Only regular blood transfusions make him like a regular child," she says. The boy has β -thalassemia, a potentially fatal genetic disorder that hampers the body's ability to produce hemoglobin.

To his parents, the boy's illness was initially confounding. Neither of his older siblings has the condition that locals call *daduzai*, or "big tummy child." The nickname belies the severity of the illness, which manifests soon after birth and claims its victims swiftly. *Daduzai* is found throughout southern China, but in Guangxi Zhuang Autonomous Region, which borders Vietnam, it is a particularly terrifying threat. Roughly one in five people in Guangxi carry a gene for one of the recessive thalassemia blood disorders; 5% of residents have a gene for a β -thalassemia disorder. Among that subset are the boy's parents. But they didn't realize that they are carriers, which meant that any child they conceived had a one in four chance of developing the disease.

Worldwide, an estimated 63,000 children a year are born with β -thalassemia, most of them in Southeast Asia and the Mediterranean. The exact number of cases in China

is not known. But it is clear that the disease strains health resources: In addition to monthly transfusions, patients may need regular iron chelation therapy through pills or a mechanical pump. Treating a baby costs up to \$4900 a year, with fees increasing as the child grows. (Patients have been cured with stem cell transplantation, but the high cost and scarcity of suitable donors puts it out of reach for most people.) To avoid having a baby with thalassemia, couples in endemic areas may undergo prenatal screening and abort fetuses with two copies of the gene.

To ease the disease burden, the Chinese government has embarked on a comprehensive prevention program featuring population screening, prenatal diagnosis, and genetic research funding. The goal is to do as well as a program in Cyprus that reduced β -thalassemia incidence from 1 per 158 births in the 1970s to close to zero today. "We want to reach every couple," says Zhang Xue, a medical geneticist at Peking Union Medical College and the Chinese Academy of Medical Sciences (CAMS) in Beijing who is involved with the Chinese initiative.

Combating the disease in Guangxi, one of China's poorest provinces, could prove challenging. The mountainous region is inhabited by nearly a dozen ethnic minorities spanning a range of cultures and languages. Thalassemia is a particular problem for the Zhuang, Guangxi's largest minority group. Their insularity contributes to the disease's persistence, says Chen Lili, director of maternal and child health for Guangxi provincial health department: "There is a lot of intermarriage."

Fresh blood. Many thalassemia patients need monthly transfusions to stay alive.

Researchers at Guangxi Medical University and CAMS first performed prenatal diagnosis here for α -thalassemia in 1983, and for β -thalassemia in 1987. Chen Lili, like most others in the health professions, was not familiar with the disorder in the 1980s when she was a young doctor. She was educated in an awful way: assisting on a birth in which thalassemia killed both the mother and baby.

Back in the 1980s, attempting to combat a genetic disease like thalassemia was thought to be too costly. But by 2010, simple testing technologies available in the wake of the Human Genome Project made community genetics possible, Zhang says. That year, the Chinese government set three targets for Guangxi by 2015: Reduce the severe thalassemia birth rate by 50%, screen all newlyweds and pregnant women, and train more than 10,000 health professionals to recognize genetic diseases. The provincial and central governments have so far spent more than \$80.7 million on the drive. Guangxi health officials say that from 2010 until now, they have screened 4.2 million people in a population of 46.1 million.

Maintaining that momentum will be key. A similarly ambitious program in Thailand has faltered for lack of political will, says Suthat Fucharoen, a hematologist at Mahidol University in Bangkok. In 2006, Thailand screened all pregnant women for the disease. But then the health department changed hands and the program effectively ended. Today, Fucharoen says, "We have no data." Getting thalassemia under control in China, he adds, will require strong central government support after the allotted 5 years and an educational campaign reaching beyond Guangxi and neighboring provinces. "In 5 to 10 years they may have very good results in southern China," Fucharoen says.

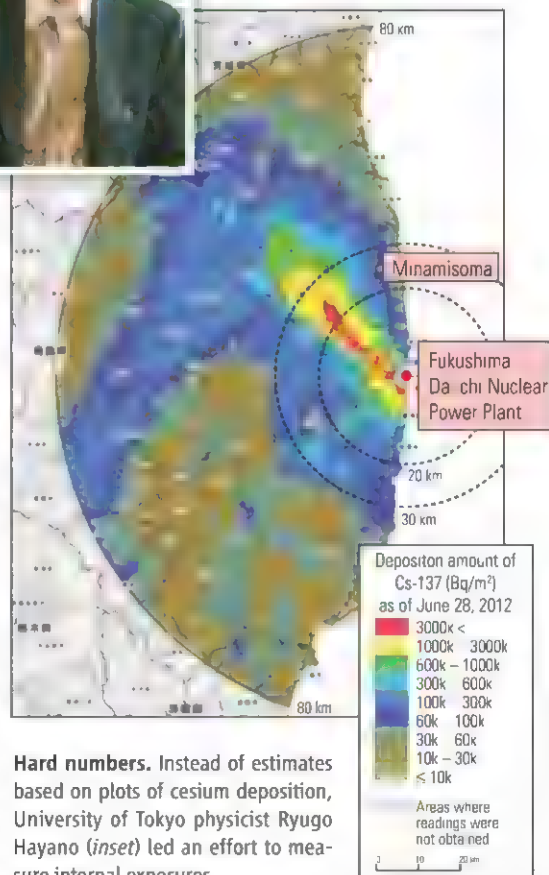
Cypriot model, Chinese characteristics

In the late 1960s, chemist and Nobel laureate Linus Pauling suggested that carriers of genetic diseases like thalassemia refrain from marrying—and, to ward off genetically risky love affairs, tattoo their DNA status on their forehead. Taking on thalassemia in the 1970s, Cyprus didn't go quite that far. But the island introduced universal testing, intended to prevent marriage between carriers, along with prenatal screening once it became available. Because screening is often linked to abortion—and the island's influential Orthodox Christian church opposes abortion—some observers were surprised by the program's

-MARA HVISTENDAHL

Insistence on Gathering Real Data Confirms Low Radiation Exposures

Hayano takes compliments and complaints in stride. "I've asked myself many times, 'Why am I doing this?'" he says with a wide grin. Others are happy to answer that question for him. "The government and the experts in this field lost people's trust due to their lackluster performance after



Hard numbers. Instead of estimates based on plots of cesium deposition, University of Tokyo physicist Ryugo Hayano (*inset*) led an effort to measure internal exposures.

Hayano is an unlikely hero. Since 1997, he has led the ASACUSA antimatter experiment at CERN, the European laboratory for particle physics. His work on antiprotonic helium atoms netted him the 2008 Nishina Memorial Prize, Japan's most prestigious physics award. He never expected to become

an expert in radiation studies. Launching one initiative after another in Fukushima, Hayano says, "I kept thinking that someone better qualified to do this will show up and take over."

When the Japanese government declared a nuclear emergency on 11 March 2011, about 5 hours after the earthquake, Hayano started keeping an eye on online radiation data streaming from a monitor near the Fukushima power plant. On 12 March, a hydrogen gas explosion rocked one of the reactor buildings. Early the next morning, Hayano tweeted a simple graph showing how radiation spiked at the time of the blast. He started posing questions to educate his Twitter followers: "Why does cesium emit gamma rays?" And, "How do you measure internal radiation?" He would note the correct answer and add a bit of explanation. Within less than a week, he went from 3000 to 150,000 followers.

The education process worked both ways. From his followers, Hayano picked up on concerns about contamination in school lunches, despite government assurances that radioactivity in all food going to market was under 100 becquerels per kilogram, a more conservative threshold than most countries. With the cooperation of Minamisoma, a city straddling the evacuation zone north of the stricken plant, Hayano once a week since January 2012 has had everything from a lunch tray at a grade school and at a nursery school thrown into a blender and tested in a highly sensitive detector, rarely finding samples exceeding 1 becquerel per kilogram. At first, Hayano shelled out for the testing himself, spending about \$3000 in 3 months until a government grant kicked in. Twitter followers started sending contributions, as much as \$1000, which he has used to "cover everything I do in Fukushima."

Even before his school lunch program, Hayano's tweets were attracting the attention of Fukushima area physicians. Since the disaster, Tsubokura has been volunteering twice a week at Minamisoma City General Hospital, which was left short-handed when doctors and nurses fled the region. In summer 2011, they started measuring radiation in concerned residents. But the results were strange, Tsubokura says. With Hayano's help, they figured out that the whole body counters the hospital was using were not shielded from background radiation.

After getting testing programs in shape, Hayano and his collaborators gathered inter-



On the road.
Solar-powered monitors track radiation near Minamisoma schools

nal exposure data for more than 32,000 Fukushima residents. Their report, published online on 11 April in the *Proceedings of the Japan Academy, Series B*, indicates that none of the 10,200 children under age 15 who were given individual whole body scans at a hospital with the most advanced, shielded counters between May and November 2012 had detectable radiocesium in their bodies. (The tests focused on cesium-137, which has a half-life of 30 years. Another isotope also released from the Fukushima reactors, cesium-134, has a half-life of about 2 years.) Only four adults had levels that would cause worrisome annual radiation doses. The researchers traced the radiocesium in those adults to wild mushrooms and wild boar meat they had obtained themselves, bypassing mandatory testing of food sold in markets.

"The result was not totally unexpected," Ban says. Internal exposure data gathered by Fukushima Prefecture authorities, for example, also suggest that radiocesium ingestion is low but do not provide individual doses in becquerels or note the number of subjects below the detection limit. "Data in Hayano's paper are given as a cesium concentration in the body, which is more straightforward and precisely interpreted," Ban says. Hayano's monitoring, adds Peter Hill, a radiation health expert at Forschungszentrum Jülich in Germany, "provides information on the real situation" that will help validate models and dose estimates. Indeed, they may influence a draft report on Fukushima for the U.N. Scientific Committee on the Effects of Atomic Radiation due later this month. Hayano's results provide "some necessary initial data that allowed me to assess public internal doses," says Mikhail Balonov, a radiation protection specialist at the Institute of Radiation Hygiene in St. Petersburg, Russia.

Hayano's reassuring findings may be welcomed by governmental leaders, but he is

still critical of their handling of the disaster's aftermath. Virtually every Fukushima resident now wears a personal dosimeter that measures external exposure to environmental radiation. But internal and external exposure data are not being combined to assess total individual doses because of incompatible databases and privacy concerns, Hayano says. Merging the data sets would not only give a more complete picture of individual exposures, he says, but it would also be useful for the international community in studying exposure risk and preparing for future accidents.

Hayano would also like to see exposure data used to prioritize decontamination as authorities move toward allowing evacuees to return home. He has set an example here as well. Hayano and Minamisoma officials identified about 100 children with the highest external exposures, based on personal dosimeter data. They then placed in their homes custom-made radiation monitors that plug into wall sockets. Data are transmitted to a central station over mobile phone lines. They also set up solar-powered monitoring stations along the roads children take to school. With radioactive surveillance in place, they can measure the effectiveness of clean-up efforts such as washing buildings, trimming trees, and scraping off topsoil. Identifying individuals who receive elevated doses, and decontaminating their homes and surroundings, should lead to a step-by-step reduction in community exposures. At a talk here last month in Tokyo, Hayano called the current untargeted approach to decontamination "silly" and, turning to Hikariko Ono, a deputy chief cabinet secretary who was in the audience, told her "this is something the government has to work on." Ono later said she would take the matter up with aides to Prime Minister Shinzo Abe.

In the meantime, Hayano is using his ingenuity to fill another data gap. Using tracking data from mobile phone companies, he is estimating acute exposure doses of people in areas subjected to radiation plumes before evacuations were carried out. And he has assembled a team to design a scanner for babies—to provide peace of mind for parents, he says. But Hayano is also looking to wrap up his involvement, so that he can concentrate once again on antimatter physics. After the baby scanner is finished, he says, "I can say I've done enough."

—DENNIS NORMILE

LETTERS

edited by Jennifer Sills

No Excuse for Habitat Destruction

IN THE PAST, MOST LOSSES OF SPECIES HAVE BEEN THE RESULT OF ignorance or an unfortunate catastrophic event (1, 2). We now see a government in a developed nation taking calculated actions to drive an endangered species to extinction.

To counter biodiversity loss, the most widely used strategy is to establish reserves. Hence, a system of protected areas was established in 2008 for Leadbeater's possum (*Gymnobelideus leadbeateri*)—the faunal emblem of the Australian state of Victoria. This reserve system is based on a population viability analysis that uncovered the best strategy for cost-effectively securing the species (3). The strategy consists of no-logging reserves embedded within government-owned forests that are otherwise primarily for wood production (4). Yet in the past 2 years, government-sanctioned changes in legislation (5) and substantial watering down of protocols for habitat protection (6) have resulted in clearfell logging of those reserves (in which most of the trees are cut down) and destruction of known habitat for Leadbeater's possum. Clearfell logging renders the animal's forest habitat unsuitable for at least 150 years (4).

Global analyses have revealed that the formal reserve systems of

many developing countries are being degraded by logging, land clearing, mining, and other practices (7). A poor understanding of ecological and conservation requirements, together with management inaction, leads to species becoming threatened or extinct in such countries. These considerations provide no excuse in the case of Leadbeater's possum; the species has been the subject of more than 30 years of detailed research (4). Government-sanctioned legal logging of the reserve system will significantly increase the chance of extinction of Leadbeater's possum. To the best of our knowledge, and despite state and national threatened species legislation, this is the first time an Australian government has taken calculated actions to substantially reduce the viability of an IUCN-listed endangered species with full knowledge of the likely consequences.

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Unpalatable Politics

THE "CONSOLIDATED AND FURTHER Continuing Appropriations Act of 2013" (1) guaranteeing funding for the federal government has, buried in the legislation, a direct attack on science. Senator Tom Coburn (R-OK) introduced an amendment that eliminates all political science research funded by the National Science Foundation (NSF), except where it promotes the "national security or the economic interests of the United States" (2). The amendment passed under a voice vote in the Senate, the full bill passed both the Senate and the House, and the

President signed it into law on 26 March.

While a seemingly innocuous bit of legislating, this amendment constitutes a serious threat to the conduct of science in the United States. The NSF has long been a preeminent institution for funding basic research and relies on an independent peer-reviewed system. Now political judgment is supplanting scientific judgment. The congressional mandate is clear: No funding will be available for basic research in political science. Legislation now dictates which topics can be studied and eliminates entire fields of study.

Some scientists may view this as a minor matter. After all, some believe that the study

of politics cannot be scientific or that this is simply one small program among hundreds at NSF. However, political science is a defined discipline. It studies the exercise of power, it tests hypotheses, and it draws inferences from well-measured empirical phenomena. Worse, the larger science community should not ignore the shackling of one program at NSF. If politics dictates what is worth studying, all disciplines are at risk. Why stop at political science? Why not neuter any grants that touch on evolutionary theories? After all, many in Congress deny the value of Darwin. The challenge to science is clear. If politics determines what is palatable,

CREDIT: DAVID LINDENMAYER

we could be picked off one at a time. The science community needs to clearly voice its opposition to this political intrusion in defining what is acceptable science.

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Don't Cull Wild Birds Yet

WITH THE LATEST THREAT OF THE NOVEL H7N9 avian influenza in China, poultry are being extensively culled in an attempt to stem the outbreak (1). Although part of the genetic makeup of the H7N9 virus is suspected to be from a group of avian viruses circulating in wild birds (2), there is no evidence that wild birds are the final animal hosts for reassortment into human-infectious H7N9. In fecal samples collected from wild birds over the past few weeks, no H7N9 virus has been found in wild birds, save for a solitary pigeon

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Deadline for submissions is 17 May. A selection of the best responses will be published in the 5 July issue of *Science*. Submissions must be 250 words or less. Anonymous submissions will not be considered. Please submit only once.

("Despite large research effort, H7N9 continues to baffle," M. Hvistendahl *et al.*, *News & Analysis*, 26 April, p. 414). Current evidence suggests that the virus's usual mode of transmission to humans involves close direct contact with domesticated birds (3). Because of lack of public awareness about the contagion, calls to cull wild birds will likely surface soon, as they did in the outbreak of H5N1 in 2004.

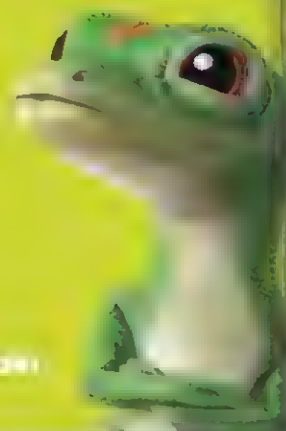
During the H5N1 outbreak, the media negatively stigmatized wild birds, leading to many ill-informed attempts by various

authorities and the public to cull wild birds or wipe out habitats to stop its spread (4). Hunters were commissioned to kill or chase away migratory birds, apparently to minimize disease transmission (4). Certain governments even revived proposals to drain wetlands to prevent migratory birds from arriving in their countries (3). However, culling wild birds does not effectively contain the disease. It may even facilitate its spread because habitat loss can stress the birds, potentially making them more vulnerable to the disease (5). In addition, infected individuals may

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disperse and spread the disease to new localities, increasing risk of disease transmission between wild and domesticated birds (4). More feasible approaches to combat the disease are to minimize interactions between domestic and wild bird populations through isolation to limit poultry infections, and to halt the live trading of wild birds (6, 7).

Calls to cull migratory birds will resurface if the current H7N9 outbreak is not contained by the start of the next avian migratory season. Let us learn from experience and prevent such a tragedy from repeating.

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TECHNICAL COMMENT ABSTRACTS

Comment on "Evidence of Abundant Purifying Selection in Humans for Recently Acquired Regulatory Functions"

Phil Green and Brent Ewing

Ward and Kellis (Reports, 28 September 2012, p. 1675; published online 5 September 2012) found altered patterns of human polymorphism in biochemically active but non-mammalian-conserved genomic regions relative to control regions and interpreted this as due to lineage-specific purifying selection. We find on closer inspection of their data that the polymorphism trends are primarily attributable to mutational variation and technical artifacts rather than selection.

Full text at <http://dx.doi.org/10.1126/science.1233195>

Response to Comment on "Evidence of Abundant Purifying Selection in Humans for Recently Acquired Regulatory Functions"

Lucas D. Ward and Manolis Kellis

Green and Ewing propose corrections to our methodology, which we incorporate and extend here. The improved methodology supports our initial conclusion of extensive lineage-specific constraint concentrated in ENCODE elements. We clarify that our estimate is dependent on the constrained and neutral references used, which can further increase the number of nucleotides involved, because a particularly stringent definition was initially used.

Full text at <http://dx.doi.org/10.1126/science.1233366>

Letters to the Editor

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Microbiomics: The Germ Theory of Everything

Fast, cheap DNA sequencing technology now allows scientists to study unculturable microbes; the results are challenging some of biology's most fundamental notions.

See full story on page 763.

Upcoming Features

Proteomics: MALDI Imaging—May 31
Data Management: Cloud-Based—June 14
Separation Techniques—July 12

FILM: ENVIRONMENT

On Rivers, Flowers, Fruits, and More



Again this March, the Environmental Film Festival in the Nation's Capital offered viewers an opportunity to sample works that advance "environmental understanding through the power of film." The 21st annual festival comprised an all-time high of 190 documentaries, stories, animations, and children's films from 50 countries. Most of the films were released in the past couple of years. Many highlighted the importance and vulnerability of rivers. The festival's website (www.dcenvironmentalfilmfest.org) includes short descriptions of all; here our reviewers comment on nine.

Trash Dance. Andrew Garrison, director. Panther Creek Pictures, USA, 2012. 68 minutes. <http://trashdancemovie.com/>

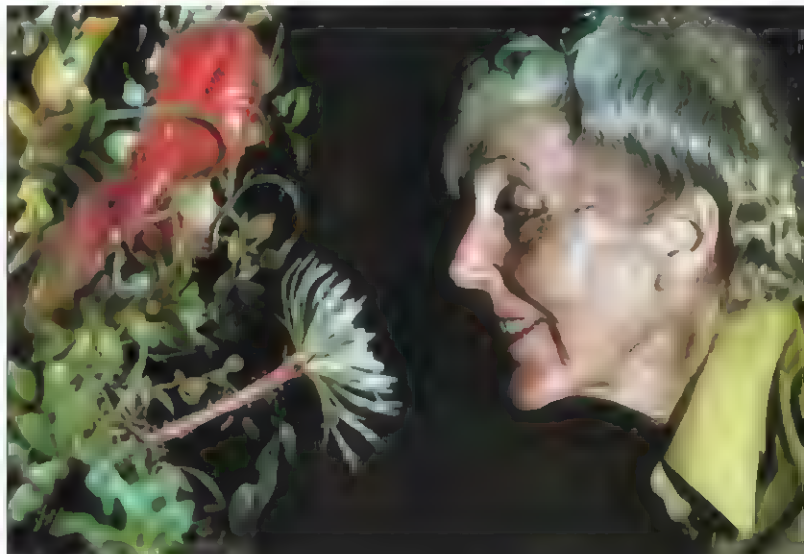
Choreographer Allison Orr spent a year (from the fall of 2008) with employees of the Solid Waste Services (SWS) department of Austin, Texas, learning about them and their jobs. Her efforts culminated in the collaborative Trash Project: a choreographed performance featuring 24 people and 16 vehicles from SWS, as well as composer Graham Reynolds and other musicians.

In *Trash Dance*, filmmaker Andrew Garrison strikes a nice balance between revealing the workers' lives and following the progress on the Trash Project. He shows Orr accompanying various specialized teams (such as household yard waste and dead animal collection) on their routes. The workers are initially suspicious of Orr; some admit to being uncomfortable with her frequent, often probing questions. As they come to trust her, they see the project as an opportunity to highlight the skills and labor involved in their underappreciated jobs.

Most SWS workers have second jobs, and many are single parents, yet they gave up their free time for rehearsals (when temperatures frequently exceeded 38°C). Some contributed individual segments—a rap, a dance solo, a harmonica song—in addition to general input on choreography. In a scene that reflects how close Orr and the performers became, they console her when a local station airs a complaint about the Trash Project and SWS reduces the number of available trucks.

Despite heavy rains the weekend of the Trash Project performance, over 2000 people wanted to see it. Unfortunately Garrison does not include the entire show, opting instead for brief excerpts that make it difficult to see how all of the parts fit together. Two scenes stand out: the show's opening segment, when a lone worker using a grabbing tool to pick up bits of trash generates applause and cheers, and after the performance, when the audience enthusiastically surrounds the workers, seeking photographs and autographs.

— Trista Wagoner



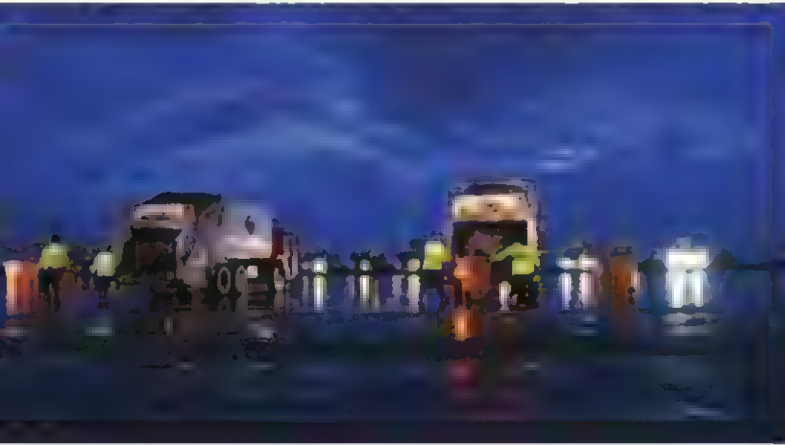
Margaret Mee and the Moonflower. Malu de Martino, director. EH Filmes, Brazil, 2012. 80 minutes. <http://margaretmee.ehfilmes.com.br/index.html>

Like the work of its titular subject, Malu de Martino's documentary is a study in color, line, and liveliness. It places the life and vitality of botanical illustrator Margaret Mee against the backdrop of the rapidly disappearing flora of the Amazon. Through a play of cinematographic and narrative effects, we glide along the *igapós* (flooded forests) of the Amazon Basin. Breathtaking scenes portray the entangled world between sky and water, day and night, forest and river.

Likewise, the viewer witnesses a dynamic interplay between Mee's incredible watercolors and the natural world that inspired her. We learn that her quest to capture the spirit of the Amazon had multiple causes: She felt inextricably bound to the place and its wilderness. And she felt bound to protect it by all the means she had—through art, science, and political speech. At times, Mee's creations seem to work their magic and outdo Mother Nature.

Born in 1909, Mee grew up in England and studied modern art. She arrived in Brazil in the 1950s for work as a teacher. Not long after, Mee took up botanical illustration and, at age 47, took the first of her 15 treks into the rainforest. The film's title calls attention to the last such trip for Mee, 40 years later, in which she completed a long-delayed quest to capture a portrait of a night-blooming cactus.

The first half of the film reveals Mee's immersion in the forest and botany, honing her own painting style and working with botanists all over Brazil to catalog and preserve plants. Midway through, de Martino switches gears to focus on the changes Mee noticed in her many trips to the Amazon—



CREDITS (TOP TO BOTTOM): JAMES WORRELL/GETTY IMAGES; SOUTH AMERICAN PICTURES/TONY MORRISON/AMX047652; COURTESY OF ANDREW GARRISON

clearcutting, the disappearance of rare plant species, and the widespread destruction of the forest itself. We see the lengths to which Mee went to engage in politics, speaking out about the threats to the Amazon to anyone who she thought might be able to do something about them. An early force in Brazil's conservation movement, she even made changes to her painting style, adding in background foliage to illustrate the importance of the forest as a whole rather than isolated plants. The film peaks with her successful trip to capture the moonflower for posterity, and it closes with a parade honoring her long-lasting legacy in Brazil.

The movie's many elements—footage of the forests and rivers of the Amazon Basin, readings from Mee's journals, recreations of trips and painterly settings, and interviews with friends and coworkers—work well together. The highlights include brief appearances by Mee herself, in an interview recounting her adventures and in footage of her final trek. However, the film seems to miss something crucial about Mee. The interviews thoroughly describe Mee's actions, personality, and passions, but we do not get a sense of how her relation to the Amazon developed. The filmmakers seem satisfied to depict her paintings, work in conservation, and deep connection with a disappearing world as complete in and of themselves. Perhaps this is because the film ultimately grapples with the memory of losing a loved one. The film instills viewers with love for both Mee and the world she cared for, and we suddenly miss things that we may have never known.

— Sarah Crespi

Peak. Hannes Lang, director. unafilm, Germany, 2011. 91 minutes. www.peak-the-movie.com/

Despite dire alarms about looming impacts of anthropogenic climate change, many of us remain relatively insulated, not yet feeling the brunt of such shifts in our daily lives. Surely some changes, in some regions, may be more rapid and noticeable than others. But changes may also be obscured by human efforts to fulfill increasingly unsustainable expectations. In *Peak*, Hannes Lang offers a hypnotizing and haunting glimpse into communities in the Tyrolean Alps, where climate, cultures, economics, and technologies are driving what one interviewee describes as “the work of pioneers” in attempts to maintain traditions and incomes in the face of global warming.

Lang artfully chooses to let stunning photography and soundscapes tell most of the story, of mountains and seasons and our attempts to live among them: humming webs of pipes and motors crisscross stark winter snowfields; verdant summer hillsides buzz with insects and the chatter of grazing livestock. Brief monologues from farmers and ski resort workers, spanning languages and perspectives from across the region, punctuate these scenes. Decrying tens of meters of glacier retreat, a resort engineer describes (and Lang shows) efforts to cover glaciers with tarps to slow melting and to construct a massive reservoir to collect snowmelt to feed increasing early season artificial snow-making. The snow-making equipment comes from a company that also manufactures desalination systems, highlighting that adaptation to inhospitable climates is neither new nor devoid of economic opportunity.



Nearby agricultural communities, having fewer resources to invest in adaptation, fear that changes in weather and grazing pastures make it harder to eke out a living off the land. All of the stories hint that physical changes in climate are largely felt through interactions with accompanying changes in regional demographics and economies—fewer young people remaining in the region and dependence shifting from agriculture to tourism.

The director wisely avoids interjecting scientific data and rhetoric, maintaining focus on the perceptions and beliefs of the people in the communities, many of whom “take with a grain of salt” what they see as climate researchers and media exaggerating “extreme data.” He elegantly closes the film with an elderly farm woman recounting the dwindling populations and livelihoods, and human-induced damage, in the region she has called home for decades. She concludes, “we just have to adapt.” With these words lingering in our ears, traditional folk harmonies accompany a slow pan out to another stunning view of a summer valley whose future remains unclear.

— Brad Wible



The Last Ocean. Peter Young, director. Last Ocean Charitable Trust, New Zealand, 2012. 85 minutes. www.lastocean.org

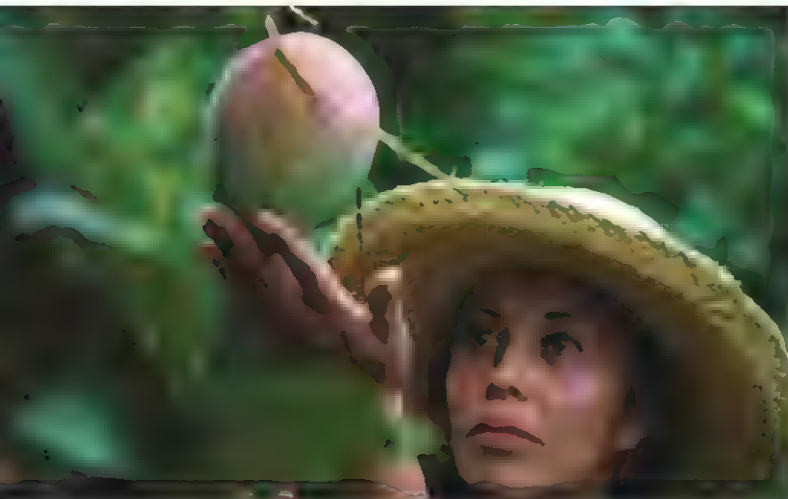
Peter Young's incisive and emotive documentary makes the case against commercial fishing in the Ross Sea, off Antarctica. Situated in the farthest reaches of the southern Pacific Ocean, about 4000 kilometers from New Zealand, it has been described as the last intact marine ecosystem on Earth—an area that can serve as a model for how other marine ecosystems used to work. Because human influence has been so limited there, top predators haven't been decimated as in other parts of the oceans.

The sea and its shores are home to abundant populations of Adélie and emperor penguins, Antarctic petrels, minke and killer whales, and Weddell and leopard seals. The top predatory fish, and prey to the whales and seals, is the Antarctic toothfish (*Dissostichus mawsoni*). Well adapted to its ice-cold environment, it produces an antifreeze glycoprotein, and its heart can beat as little as once every 10 seconds. The fish can also grow to lengths of 2 meters and weights over 80 kilograms, which makes it the subject of considerable commercial interest—so much so that boats from several nations have been fishing it, under treacherous ice conditions, since the late 1990s. Like its close relative, the Patagonian toothfish, Antarctic toothfish is sold as “Chilean sea bass,” a prized delicacy that can bring as much as \$70 per kilogram.

The film gives voice to the scientists who study the Ross Sea ecosystem and to all of those who would like to keep it mostly untouched, as it was before commercial fishing arrived there. Poignant and poetic as well as informative, *The Last Ocean* forms part of a wider campaign to create a marine protected area that covers the whole Ross Sea ecosystem.

— Maria Cruz

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The Fruit Hunters. Yung Chang, director. EyeSteelFilm, Canada, 2012. 95 minutes. www.eyesteelfilm.com/fruihunters

Although the term “luscious” is not often used to describe a film, in the case of *The Fruit Hunters*, it turns out to be apt. With sumptuous cinematography and a seductive soundtrack, Yung Chang’s captivating film introduces us to the world of exotic fruit and an assorted cast of characters who have made fruit their life’s passion. We meet several of these characters at the Rare Fruit Council International Conference in Homestead, Florida. (\$800 may seem pricey for a vintage bottle of wine but imagine paying this for a white mango, as one eager conference attendee happily did.) We also meet movie star and fruit connoisseur Bill Pullman, who has a backyard orchard of exotic fruit trees at his home in the Hollywood hills. The film follows Pullman as he plans with his neighbors to develop a community orchard on a small rock outcropping that is up for sale near his home, a plan that is ultimately foiled when the land is sold to a developer.

But the film’s true star is Colombian forester Noris Ledesma, who travels the world collecting cuttings of rare fruit trees to expand the genetic collection at Florida’s Fairchild Tropical Botanic Garden. Passionate about saving what’s left of the world’s diminishing biodiversity, Ledesma was forced to flee her country due to continuing conflicts in the Amazonian region where she worked. We travel with her from Bali to Borneo as she chats with local women selling fruit at the markets to discover the locations of rare fruit trees so that she can obtain precious cuttings. In search of the prized kura kura (durian) fruit, Ledesma follows an elder of the Penan tribe deep into the heart of the Bornean rainforest. She learns that with half of the rainforest already destroyed to make way for a monoculture of oil palm trees, the kura kura and the Penan’s traditional way of life are in jeopardy.

Ranging from durian and jack fruit to tampoi, dragon fruit, and the miracle berry, this film reveals the astonishing variety—every conceivable color and shape—of fruits on planet Earth. Anyone who has seen this delectable film is unlikely to view a piece of fruit, whether common or exotic, in the same way again.

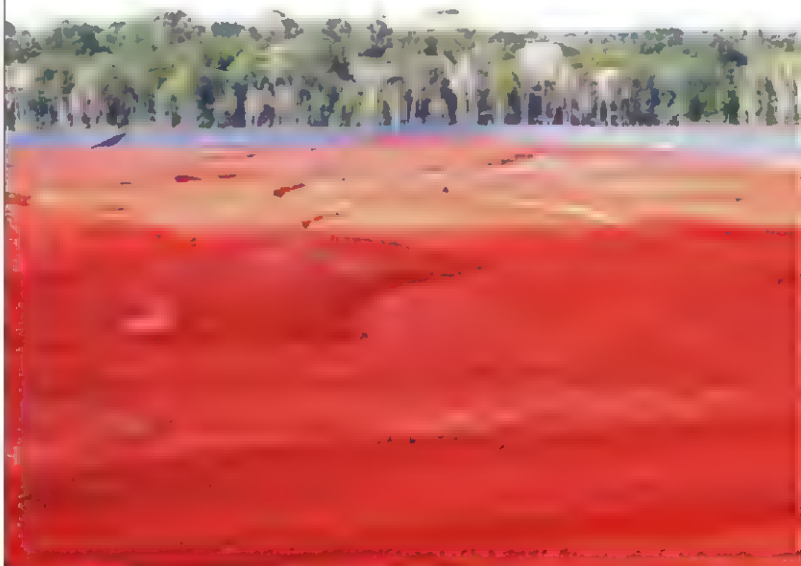
— Orla Smith

The Age of Aluminium. Bert Ehgartner, director. Langbein & Partner Media, Austria, 2013. 90 minutes. www.langbein-partner.com/en/?p=379

Aluminum (or aluminium) is an odd element, and not just because it has multiple English spellings. Of the most common elements in Earth’s crust, it is the only one to have no known beneficial biological function. Humans, however, have certainly found a myriad of uses for aluminum otherwise, from building materials to drug additives. What *The Age of Aluminium* aims to point out is that we may not fully realize or understand the implications of our increased reliance on this unusual metal.

Bert Ehgartner’s film mostly alternates between exploring aluminum’s environmental and public health consequences, both of which the film implies are tragically underappreciated. Take, for instance, the impact felt in regions where aluminum mining is prevalent, most of which happen to fall in developing countries. Deforestation and degradation of regional water quality are likely outcomes, but neither was as visually compelling as the footage of countless quantities of “red mud” produced by extracting aluminum from bauxite. Handling this waste effectively is an incredibly difficult problem, and one that the film treats carefully.

More problematic, however, are the sections of the film that deal with human health effects of chronic and acute exposures to various aluminum compounds. To be sure, the film is able to generate a strong emotional response showing cancer patients shedding tears as they cope, grieving survivors of disasters visiting the grave sites of loved ones, and Alzheimer’s patients struggling with simple daily tasks. But implicating aluminum as the cause is less convincing—especially when discussing politically charged issues like vaccines or trace contaminants in drinking water. Some of the



expert interviewees strike cautionary tones when definitively linking aluminum exposure to neurological effects, but overall the film fails to strike a balance between our scientific understanding and public concern. Perhaps the best lesson to take away from the film is not that we’re just in the age of aluminum but that we’re in the age of consumption, population growth, and globalization. There are environmental and public health risks associated with virtually any material we mine, process, eat, or prescribe. If we’re not able to understand and mitigate these risks, we’ll soon find ourselves in a new age altogether.

— Nicholas S. Wigginton

A Fierce Green Fire: The Battle for a Living Planet.

Mark Kitchell, director. First Run Features, USA, 2012. 110 minutes. www.afiercegreenfire.com/

Today, calls to protect the environment are common although, unfortunately and to our peril, often ignored. This has not always been the case, and in *A Fierce Green Fire* filmmaker Mark Kitchell takes us through the history and importance of the environmental movement in five acts. His account opens in the early 20th century with “conservation,” which describes the recognition by early environmentalists such as John Muir that wild nature is not purely for human use but is worthy of protection for its own sake. Leading us through four more movements (“pollution,” “alternatives,” “going global,” and “cli-



Seeing the environmental movement from the perspective of history can leave one feeling frustrated—particularly given an overall impression that much earlier hopeful momentum has led to little recent action despite our now facing even bigger challenges. In the end, however, the message that one takes away from the film is that of inspiration. One hopes these stories may stoke a fierce green fire in all of us.

— Sacha Vignier



Rock the Boat: Saving America's Wildest River. Thea Lucia Mercouffer, director. Magic Pebble Media and WSR Creative, USA, 2011. 52 minutes. <http://rocktheboatfilm.com/>

Lost Rivers. Caroline Bâcle, director. Catbird Productions, Canada, 2012. 72 minutes. www.catbirdproductions.ca/2010/04/22/lostrivers/

There really is a river in Los Angeles. Where? "No idea!" bemused residents reply in Thea Lucia Mercouffer's *Rock the Boat*, as well they might. The L.A. River is now little more than a huge concrete flood control channel crisscrossed by freeways. Other smaller urban rivers and creeks have fared even less well than it, as Caroline Bâcle's *Lost Rivers* chronicles: Many have been buried entirely, ignobly turned into storm drains and sewers. Both films document just how far we have bent urban rivers to our will, and both are a rallying cry to unbend them.

Don't doubt that untamed rivers are as dangerous as they are beautiful: They are unpredictable in their destructive power, most especially in the close confines of a city (water can move quickly and is heavy; a cubic meter weighs a metric ton). There is an inherent necessity in their pacification. Even so, it is clear we have been atrociously poor guardians, partly through ignorance and partly through greed—as the two films make abundantly clear.

Lost Rivers introduces us to a class of urban explorers known as "drainers," some of whom have become involved in battle to recognize sewers and drains for the rivers they once were. They are a species of spelunker, hauling up drain lids with crowbars and disappearing into the maze of underground pipes and tunnels before the authorities can nab them.

Rock the Boat chronicles the exploits of a more familiar breed of river explorers and activists: a team of canoeists and kayakers who in 2008 paddled the L.A. River to the sea, much of the way in a thin straight-edged slot in the center of the flood-control channel. They make their expedition an event, gently defying the U.S. Army Corps of Engineers, who had declared the river unnavigable (and therefore exempt from the Clean Water Act), and endearingly bemused officers of the Los Angeles Police Department.

Like many pioneering activists, the drainers and canoeists (and filmmakers) break the law to make their point. And, satisfyingly, there is a reward for their moral courage. Drainers in Brescia, Italy, have been brought under the umbrella of the local government and now provide tours of the Roman-built subterranean rivers under that town. The L.A. River voyagers were part of an ultimately successful campaign to have the river recognized as navigable and subject to the Clean Water Act.



There are other success stories. In southeast London, urban flood engineering has freed the River Quaggy from its concrete culvert and turned an urban park into a wetland that can absorb flood waters from the river in times of serious inundation, allowing water to percolate back into the ground. Rivers in Yonkers, New York, and in Seoul, South Korea, have been "daylighted" (literally exhumed) and again become havens for wildlife. They now serve as places where people can encounter the plants and animals that share the river with us.

In raising public awareness of these forgotten rivers and as portrayals of citizen activism, these films are important as well as inspiring. Their message is clear: Urban rivers can, as they did in the past, offer solutions to the problems of living in cities. If we let them.

— Guy Riddihough

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RESEARCH PRIORITIES

The NIH BRAIN Initiative

Thomas R. Insel, * Story C. Landis, * Francis S. Collins*

The NIH BRAIN Initiative will build on recent successes in neuroscience to create and apply new tools for understanding brain activity.

On 2 April 2013, President Barack Obama announced the Brain Research through Advancing Innovative Neurotechnologies (BRAIN) Initiative. In front of some 200 scientists in the East Room of the White House, the President declared, "...there is this enormous mystery waiting to be unlocked, and the BRAIN Initiative will change that by giving scientists the tools they need to get a dynamic picture of the brain in action and better understand how we think and how we learn and how we remember. And that knowledge could be—will be—transformative" (1).

Many scientists have been skeptical of an ambitious new project when support of existing science is eroding. Others have been confused about how this project will accomplish its goals, because mapping the brain is far more complex and open-ended than mapping the genome. Still others have speculated about whether this is really a new initiative with new funding, or if it simply reflects rebranding of current research. Here, we will try to address these concerns and explain in more detail the NIH vision for the BRAIN Initiative.

The BRAIN Initiative is being launched with a proposal for federal funding of just over \$100 million in the next fiscal year and will be led by the National Institutes of Health, the Defense Advanced Research Projects Agency (DARPA), and the National Science Foundation (NSF). Private partners—including the Allen Institute for Brain Science, the Howard Hughes Medical Institute, the Kavli Foundation, and the Salk Institute for Biological Studies—are also committed to ensuring its success. A preliminary vision, called the "Brain Activity Map," was initially developed at a series of meetings sponsored by the Kavli Foundation, the Gatsby Charitable Foundation, and the Allen Institute for Brain Science (2, 3). BRAIN seeks to significantly extend and shape that vision, exposing the research plan to rigorous scientific debate with broad input from the neuroscience community.

Mapping brain structure and function is already an exciting field of science. New ana-



tomic techniques, from Brainbow to CLARITY, provide unprecedented images of neural architecture (4, 5). Breakthrough technologies—such as two-photon imaging, light-sheet microscopy, and miniaturized microendoscopes, together with calcium imaging and voltage imaging—have given us the first dynamic views of how the brain encodes information in modular circuits (6–8). Optogenetics has enabled precise manipulation of circuit activity with light pulses (9).

In addition to these technical innovations exploited in experimental animals, human neuroimaging has advanced considerably in the past decade. The Human Connectome Project has increased gradient strength and improved white matter imaging to provide the first detailed "wiring diagram" with new insights into the three-dimensional organization of fiber tracts in the living human brain (10). Improvements in functional magnetic resonance imaging (fMRI) have given us better maps of human brain activity, allowing more precise localization of complex functions such as language, emotion, decision-making, and hallucinations. Even the analysis of fMRI signals from individuals who are not performing a task ("resting state" imaging) has been explored as a powerful marker of individual cognitive traits (11).

Do current technological advances render the BRAIN Initiative unnecessary? We argue

that this success is precisely the springboard needed for a new revolution. Brain function is a dynamic process with changes on a millisecond scale, but some of our most powerful current mapping techniques are static. Neural circuits involve at least 10^6 cells in a complex, recursive network, but neurophysiology has been based classically on single-cell recordings or, more recently, on small ensembles of cells. Human neuroimaging captures the whole brain in action, but each 1-mm³ voxel includes at least 80,000 neurons and 4.5 million synapses. An fMRI scan, with 680,000 voxels, is capturing local changes in blood flow and oxygen consumption—but these changes are low-resolution and slow surrogates for neuronal activity. Much of the progress over the past decade has been based on optimization of existing tools like MRI and physiological recording. Now is the time to create the next generation of tools by harnessing advances in diverse disciplines from engineering to computational science.

Of course, there are many questions about how to focus this new effort. Should we concentrate on tools for mapping the activity of the 302 neurons in *Caenorhabditis elegans* to yield a comprehensive dynamic picture of nervous system activity associated with behavior and, thereby, establish basic principles of circuit function? How would these technologies or these principles scale from 10^2

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neurons to 10^6 neurons in simple vertebrates or 10^{11} neurons in humans? Should we create a noninvasive version of optogenetics for use in the human brain, allowing functional dissection of neural circuits and enabling more precise diagnostics and therapeutics for brain disorders? What would substitute for light pulses to make this noninvasive, and how would the pulse be directed in the absence of a light-sensitive transgene? Should we create nanoscience tools to record from as many neurons as possible, scaling up modern neurophysiology by several orders of magnitude?

These kinds of questions deserve a thoughtful discussion. That is why we have invited a group of 15 external advisers, co-chaired by Cornelia Bargmann from The Rockefeller University and William Newsome from Stanford University, to lead such a discussion over the next several months (12). The charge to this group is to develop a scientific plan that will (i) identify areas of high priority (i.e., improving current tools, identifying new directions); (ii) develop some principles for achieving the goals of the BRAIN Initiative (i.e., balance between individual groups and large consortia, balance between problem-solving and technology-driven science); (iii) suggest opportunities for collaboration with foundations, industry, and other agencies; and (iv) deliver specific recommendations for timelines, milestones, and cost estimates. These 15 neuroscientists will be reaching out to a broad community of scientists. We realize that the seeds for the next generation of neurotechnologies may already be sitting in laboratories far removed from neuroscience. Optical physics, nanotechnology, organic chemistry, materials science, molecular biology, computational science, and many other areas will be vital to this effort. Our hope is that the core group of external advisers will serve as a hub to build spokes to other areas of science, creating a national interdisciplinary effort, not just a neuroscience effort.

What will the BRAIN Initiative cost? We are now spending more than \$5 billion on neuroscience at NIH. President Obama has asked for roughly \$100 million to launch the first year of this project and noted that a serious, sustained effort would be necessary. The NIH budget for 2014 will be determined by Congress and may not be final for several months. Nevertheless, NIH's intention is to commit at least \$40 million for new projects within BRAIN next year and to ramp up this commitment in subsequent years. We have asked the Bargmann-Newsome team to provide initial recommendations by the fall of 2013, to give us time to issue requests for applications to jumpstart NIH BRAIN in 2014.

In the first year, much of the funding will come from sources set aside for special projects. One of the largest contributions (\$10 million) will be from the NIH Neuroscience Blueprint, a consortium of 15 institutes and centers at NIH developed to support cross-cutting initiatives like technology development and neuroscience training. The NIH Director's Office will be another major contributor (\$10 million). The balance will be contributed by individual institutes, including National Institute of Biomedical Imaging and Bioengineering, National Institute on Drug Abuse, National Institute of Mental Health, and National Institute of Neurological Disorders and Stroke. Will this new commitment reduce support for investigator-initiated R01 grants in these institutes? This will represent less than 1% of NIH's support for neuroscience research, any impact on the payline in these institutes should be quite modest. Indeed, experience from the Human Genome Project, which was met initially with some anxiety and skepticism from NIH R01 grantees, predicts that tool development efforts empower individual labs by providing better, cheaper discovery technologies and open-access databases.

Where will NIH BRAIN live? The BRAIN advisory group will report to the Advisory Committee to the Director, chaired by the NIH director. Rather than embedding NIH BRAIN in a single institute or center, we want this project to involve many parts of NIH. That explains the important role of the NIH Neuroscience Blueprint. NIH BRAIN will also need to synergize with DARPA and NSF and their plans, as well as projects outside of government, industry programs, and some spectacular new efforts outside of the United States, such as the European Human Brain Project (www.humanbrainproject.eu/). Bridges to all of these efforts will be developed to avoid redundancy and expedite progress.

The goal of NIH's contribution to the BRAIN Initiative is to produce insights into brain disorders that will lead to better diagnosis, prevention, and treatment. The Institute for Health Metrics and Evaluation estimates that brain disorders (neurologic, substance abuse, and mental and behavioral disorders) are the number-one source of disability globally, accounting for 22% of disability adjusted life years (DALYs) from all medical causes in the 15- to 49-years age bracket (13). The World Economic Forum predicts that these same disorders will have the largest costs of chronic, noncommunicable diseases globally over the next 20 years (14). Recent estimates of the cost of Alzheimer's disease alone demonstrate the profound increase we can expect over the next few decades if we do not find a

way to preempt, delay, or treat dementia (15). Building a foundation of understanding of the emergent properties of the brain will provide an opportunity for deeper insights into Alzheimer's disease, Parkinson's disease, schizophrenia, bipolar illness, autism, epilepsy, attention deficit hyperactivity disorder, traumatic brain injury, and a long list of other brain disorders. Those clinical benefits will be the ultimate payoff of this initiative for human health—but we must be careful not to overpromise the immediacy of such outcomes.

President Obama ended his announcement of the BRAIN Initiative by saying, "I don't want our children or grandchildren to look back on this day and wish we had done more to keep America at the cutting edge. I want them to look back and be proud that we took some risks, that we seized this opportunity." The combination of public health need and scientific opportunity is really why the BRAIN Initiative is "the next great American project." As with the Human Genome Project in 1988, the starting gate is a careful, inclusive, deliberate planning process. Mapping the brain is not as simple as mapping the genome—there will be no linear sequence to decode and no obvious end point. But the lessons learned from this earlier effort—lessons about tool development, ethical implications, and partnerships—can be helpful as we launch a new, even more daunting adventure. Just as the Human Genome Project transformed biology, we predict that the BRAIN Initiative will not only transform neuroscience but will yield the intellectual and technological framework for better diagnostics and therapeutics for the millions worldwide who suffer from brain disorders.

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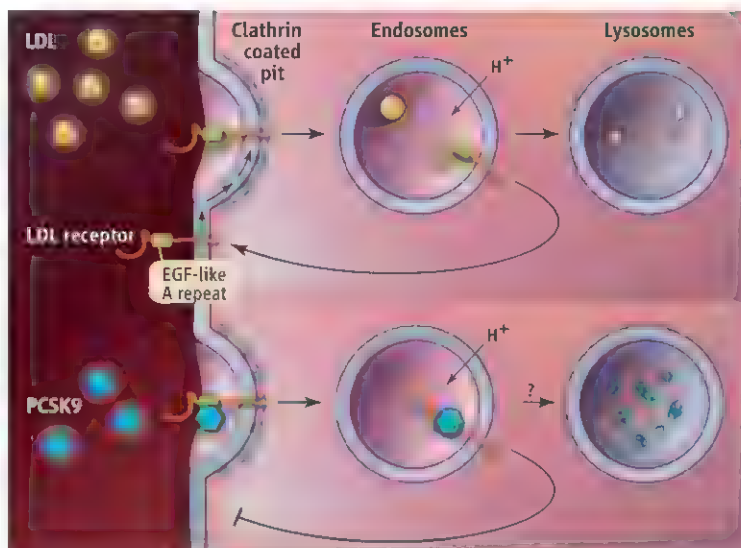
GENETICS

Simple Genetics for a Complex Disease

Jonathan C. Cohen^{1,2} and Helen H. Hobbs^{1,3}

The cost of bringing a drug to market is staggering (estimated at more than 1 billion dollars) and the failure rate is daunting: Only one in three drugs that reach phase 3 clinical trials ultimately reach the marketplace. Accordingly, there has been considerable interest in improving strategies to predict the effects of new therapeutic agents. The outcome of several recent trials of a new class of cholesterol-lowering agents—antibodies against an enzyme called proprotein convertase subtilisin kexin type 9 (PCSK9) (1)—illustrates the potential role of human genetics not only in identifying drug targets, but also in predicting the likely consequences of specific interventions.

PCSK9 was first implicated in the metabolism of low-density lipoprotein (LDL)—the so-called “bad” cholesterol—when mutations in the *PCSK9* gene were shown to cause a rare form of hypercholesterolemia (2). Subsequently, both normal and mutant forms of PCSK9 were found to promote degradation of hepatic LDL receptors (LDLRs), the primary conduit for clearance of circulating LDL (3). This suggested that the *PCSK9* mutations that cause hypercholesterolemia confer a gain-of-function. Loss-of-function mutations in *PCSK9* would be expected to increase the number of cell surface LDLRs and decrease circulating LDL concentrations. Confirming this prediction, nonsense mutations in *PCSK9* are associated with a 30 to 40% reduction in LDL concentrations and confer an 88% reduction in incident coronary heart disease (CHD) with no appar-



Rerouting. The intracellular pathways of the LDL receptor after binding LDL or PCSK9 are shown. Antibodies that target PCSK9 prevent the rerouting and degradation of LDL receptors, thereby reducing plasma concentrations of LDL. EGF, epidermal growth factor.

ent adverse effects (3). Two women with no PCSK9 and extremely low LDL concentrations (14 to 16 mg/dl) have no apparent health problems (4, 5). These data pointed to PCSK9 inhibition as a potentially effective and safe strategy to prevent CHD.

Despite much effort, no viable small-molecule inhibitors of PCSK9 have been identified. An alternative approach based on the observation that PCSK9 acts from outside of the cell in mice and in cultured cells (3) has proved to be highly effective. In six clinical trials, monoclonal antibodies against PCSK9 that block its interaction with the LDLR reduced plasma concentrations of LDL (up to 70%) with few side effects, even in patients already treated with cholesterol-lowering statins (small molecules that inhibit cholesterol synthesis in the liver) (1). Moreover, anti-PCSK9 therapy was highly effective in patients who did not reach optimal LDL amounts on statins, or who are unable to tolerate statins (1). Phase 3 clinical trials are currently under way to determine the safety of prolonged anti-PCSK9 therapy and whether the additional LDL lowering achieved by inhibiting PCSK9 confers improved protection against CHD.

Low-frequency alleles with large phenotypic effects may guide the development of therapeutics and treatments.

Another approach would be to target pathways required for PCSK9 secretion (6) or degradation. PCSK9 acts by rerouting internalized LDLRs from a pathway that returns them to the cell surface to a pathway that directs them to lysosomes, where they are degraded (see the figure). The structural features of both PCSK9 and LDLR that are required for lysosomal targeting have been defined (3), but the biochemical mechanism by which PCSK9 interdicts receptor recycling, and the pathway(s) by which LDLR-PCSK9 complexes reach the lysosome, are not known. Inhibition of established pathways to degradation in lysosomes,

including the endosomal sorting complex required for trafficking (ESCRT) pathway, does not block PCSK9-mediated degradation of LDLRs (7).

The identification of low-frequency *PCSK9* alleles with large phenotypic effects facilitated the rapid development of PCSK9-based therapy. This approach is now being advocated as a model for other complex diseases. However, it is not yet clear whether PCSK9 will be paradigmatic or exceptional. The large effects of relatively common *PCSK9* alleles on CHD reflect a remarkably linear causal cascade: LDL is the dominant determinant of CHD (8). Other risk factors such as diabetes and smoking only become prominent at permissive amounts of LDL. Circulating concentrations of LDL are profoundly influenced by LDLR activity (9), which in turn is strongly determined by the activity of PCSK9. Differences in PCSK9 activity are thus transduced through the direct coupling of LDLR-LDL to CHD risk. Such tight couplings to single genes may be incompatible with the stringent homeostatic constraints on pathways central to many other complex diseases (e.g., blood glucose, blood pressure, and inflammation).

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The *PCSK9* alleles that provided a direct path to clinical translation confer protection from, rather than susceptibility to, CHD. Identification of protective alleles is limited by several factors: Alleles that cause disease are likely to be more frequent in clinical populations, whereas protective alleles are not and must be ascertained from the general population. Proving genetic association with protection from disease is more difficult than is proving association with increased risk of disease, and is only feasible for alleles present at appreciable frequencies in the study population. Because purifying selection is a powerful barrier to accumulation of deleterious alleles, few mutations with large phenotypic effects are likely to reach the requisite allele frequencies (10).

The effectiveness of statin therapy for CHD has been firmly established, yet substantial residual risk of disease remains in treated individuals (>50% in most studies). By contrast, comparable reductions in LDL associated with *PCSK9* mutations result in

very low rates of incident CHD. This suggests that the residual risk of CHD in statin-treated individuals is not due to inadequate cholesterol lowering, or to other risk factors independent of LDL, but rather to delayed initiation of cholesterol-lowering intervention. Early intervention that ensures modest but lifelong reduction of plasma LDL may be the best approach to prevent CHD. Decreasing LDL by the requisite magnitude (~30%) can be easily achieved in most individuals by currently available agents. What then is the role of PCSK9 inhibitors?

Cost and convenience are considered the two major obstacles to large-scale use of antibodies against PCSK9. The importance of inconvenience as a barrier to antibody therapy may be overstated. Clinical experience with immunotherapy for allergy relief indicates that many patients will tolerate regular injections for years. The high cost of monoclonal antibodies (typically more than \$1000/month) is likely to be a more substantial obstacle, particularly given that one of the most powerful

and widely used statins is available in generic form for ~\$10/month. Thus, antibody-based anti-PCSK9 therapy is likely to be targeted at individuals at high risk for CHD in the near term, and to those who do not achieve satisfactory LDL concentrations with, or are unable to tolerate, conventional cholesterol-lowering agents. A broader role for PCSK9-based therapy may have to await the development of small-molecule inhibitors.

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PALEONTOLOGY

Feathers Before Flight

Julia Clarke

Feathers are branched structures consisting of β -keratin—a rigid protein material formed by pleated β sheets—with a hollow central shaft. They are strikingly different from other forms of vertebrate integument such as scales, skin, and hair. Until recently, evolutionary hypotheses envisioned their origin through elongation of broad, flat scales driven by selection for aerial locomotion such as gliding or flapping flight. Over the course of the past two decades, fossil discoveries, especially from northeast China, have revealed that the early precursors of feathers were filament-like rather than expanded scales and that branched pinnate feathers of modern aspect predate the origin of active flight. The revolution in our understanding of feather evolution continues, driven by rapid fossil discoveries and by new information from the study of extant birds.

One of the most transformative ideas to affect understanding of living birds has been the recognition of their perch within the tree of life on branches crowded with their extinct

dinosaurian cousins. This insight came first from comparisons of bones, the most commonly preserved part of a fossil vertebrate. Fossilized soft tissues are only preserved in a few exceptional places (Lagerstätten). The Chinese deposits provide one such unique snapshot, where over a thousand specimens with fine details of soft tissues such as feathers, hair, and skin are preserved in ash-rich lake deposits ranging from the Late Jurassic (~150 million years ago) to the Early Cretaceous (~120 million years ago). Fossils from these deposits have revealed that dinosaurs that were inferred from bone characteristics to be closely related to living birds also share more features of feather structure.

The closest theropod dinosaur relatives of birds have pinnate feathers; more distantly related theropods have simple filaments or bunches of filaments of varying lengths and diameters (1, 2). The latter forms do not fit the hypothesis of flat scales morphing directly into flat feathers. But these hollow filaments or “protofeathers” are similar to structures seen early in feather development; a simple hollow cylindrical sheath arises first in feather ontogeny from the collar of the feather follicle before the barb ridges, linked to the devel-

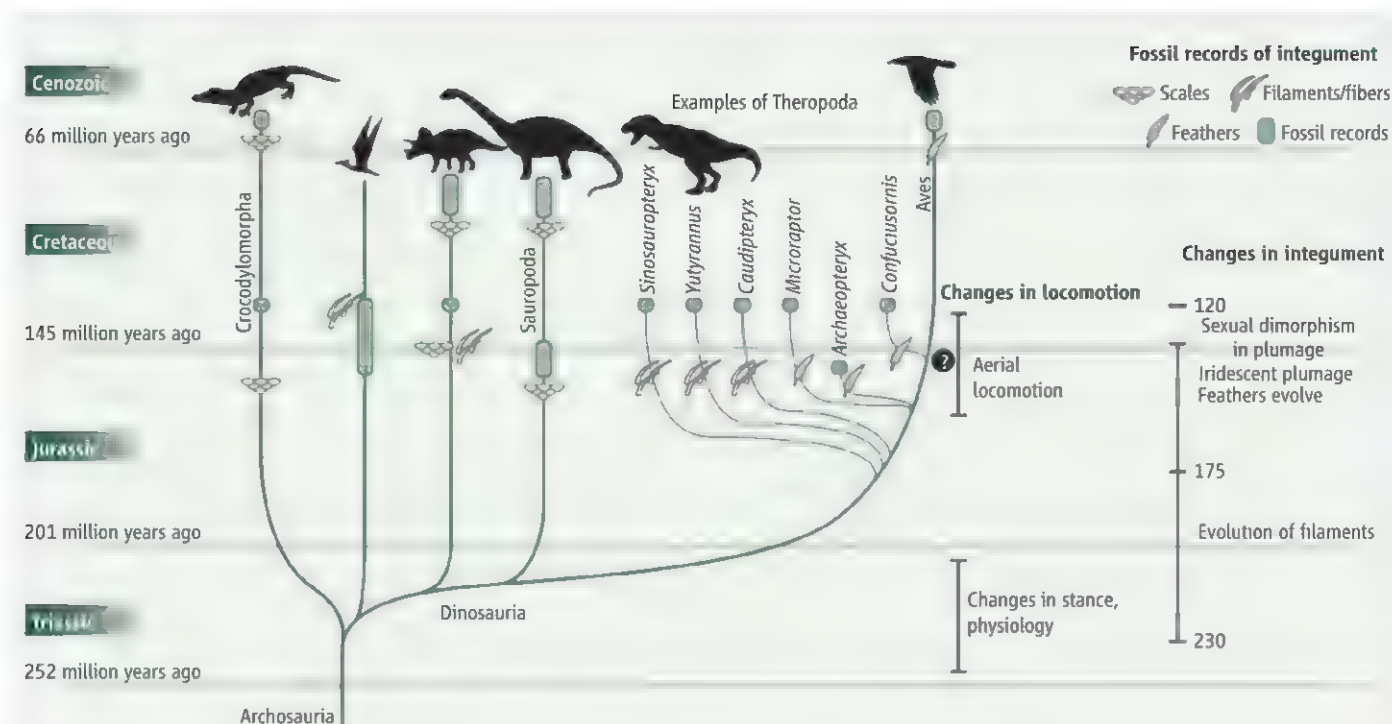
Fossil data indicate that feathers and their precursors may have evolved over a much longer span than previously thought.

opment of its branching shape, form (3). Fossil data indicated dramatic shifts from scale to filament, to bunches of filaments, to branched feathers in theropod dinosaurs. In the lineage of dinosaurs including birds, *Tyrannosaurus rex*, and many small raptors, filament- and feather-bearing species were common.

The more recent discoveries of a basal ornithischian dinosaur with a filamentous body covering, and another ornithischian more closely related to *Triceratops* with a bristle-covered tail, force reconsideration of the timing of this transition. These fossils indicate that filamentous structures may be ancestral to dinosaurs (4). Filaments called pycnofibers also covered some pterosaurs (5). Ornithischian dinosaurs, sauropod dinosaurs, and pterosaurs are on evolutionary branches that split from that of theropod dinosaurs and birds about 230 million years ago in the Triassic. If these structures have the same evolutionary origin, a form of filamentous integumentary structure evolved from scales nearly 100 million years before the locomotor transition that we call the origin of birds (see the figure).

The recent fossil data suggest that key integumentary shifts might be related not

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Preserved evidence of archosaurian body covering. The earliest preserved scales, filaments, or feathers are from the late Jurassic; the earliest crown clade bird with feathers is from the Paleocene. Filamentous feather precursors may have originated nearly 100 million years before the origin of flight, but very few fossil deposits sample this period. Sexual dimorphism in plumage and color patterning in Late Jurassic and Early Cretaceous dinosaurs suggest that display functions played a key role in the early evolution of pinnate feathers.

to flight but to innovation in stance, terrestrial gaits (6), and life history (7) in early archosaurs, which came to dominate terrestrial ecosystems by the end of the Triassic. However, there are unanswered questions. Were there at least three independent and convergent shifts from scales to filaments in Archosauria, with only one of these linked to the origin of feathers and flight? Or was there a single ancient origin of filaments, with subsequent losses in some species and, much later, a second period of novelty seen in the evolution of a branching feather form? Answering these questions is key to understanding the evolution of feathers and other integuments.

These questions send paleontologists back into the field. Early fossils of most major archosaur lineages are known from records in the Late Triassic and Early to Middle Jurassic (~225 to 165 million years ago). However, no dinosaur older than the Late Jurassic has been recovered with preserved integuments (scales or feathers). Early pterosaurs are virtually

unknown in the fossil record; their earliest fossils with integuments are also Late Jurassic in age (see the figure). A Late Triassic or Early Jurassic site with fine-scale soft tissue preservation would offer crucial insight into this question. However, very few candidate sites are known.

The fossil snapshots that we do have offer much more insight into the evolution of pinnate feathers seen in living birds. There is no known analog of archosaur filaments in adult living animals, but bird feathers are known to have diverse functions, for example, in flight, display, camouflage, and heat retention. Iridescent feathers, long tail feathers, large color patches, and dimorphism are all linked to sexual selection in living birds. Recent findings suggest that early pinnate feathers also played a role in sexual selection. Investigation of the shape and form of fossilized melanin-containing organelles (melanosomes) has indicated that the forelimb and hind limb feathers of one maniraptoran dinosaur were patterned with large conspicuous patches of white with black spots (8). Inference of plumage color in the “four-winged” maniraptoran *Microraptor* yielded evidence of glossy or weakly iridescent feathers (9). Long tail feathers are known from many species during the transition to powered flight (9–11). Finally, evidence of sexual dimorphism in early birds was recently confirmed by recovery of a bone tissue unique to reproductively active female birds in a *Confuciusornis* specimen with feathering; specimens with long tail

feathers were males and those with short tails, females (11).

Evidence is thus accruing for the function of early pinnate feathers in sexual selection, but there is little consensus on shifts in feather function associated with the evolution of flight. Reconstruction of ancestral conditions for the bird lineage requires consensus on the evolutionary relationships of key species. These species differ in feather shape as well as in their organization and layering on the forelimb and hind limb (12, 13). Whether observed differences can presently speak to a gliding or flapping origin for flight is debated. Species with elongate feathers or a “wing” on the hind limb show characters consistent with a form of aerial locomotion but not one seen in living birds (14). At the same time, continued research indicates a broader variety of locomotor functions for forelimb feathering in living birds other than powered flight; young living birds flap short pinnate feathers on the forelimb, increasing traction to climb highly inclined surfaces (15). Although historically, feathers were firmly linked to flapping flight, the evolution of their early locomotor function in climbing, taking off, turning, landing, gliding, or flapping is a key outstanding question.

The evolution of feathers is now seen as one part of a broader story concerning the origin of novel integumentary structures in archosaurs, although data on the early parts of this story are very limited. New data multiply the set of questions we must ask about

the locomotor transition that we call the evolution of flight. Model-based approaches are needed to explore the varieties of aerial and nonaerial locomotor strategies that extinct dinosaurs may have employed. These must take into account not only the diverse locomotor strategies in living birds but also potential differences in feather properties, shape, and plumage organization.

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IMMUNOLOGY

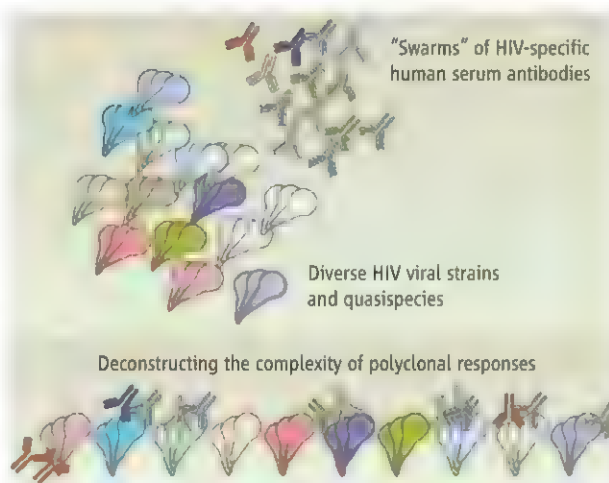
Crowdsourcing Immunity

James E. Crowe Jr.

Many viral pathogens exhibit a dramatic variability in the sequence and structure of surface proteins that are targets of the humoral immune system, allowing them to evade inhibition by neutralizing antibodies. This constant shape-shifting generates swarms of viruses in infected hosts that are often termed “quasi-species,” especially for agents such as HIV that cause chronic infections. On page 751 of this issue, Georgiev *et al.* (1) show that humans use complex and varied swarms of their own to respond to virus infection. Previously, it had been technically challenging to study the complex mixtures of antibodies found in serum in order to understand the clonal components of natural immune responses. These investigators address this with an approach that marries large-scale bioassays with computer algorithms.

Antibody engineers have recently made substantial progress in developing technologies for isolating monoclonal antibodies to viral pathogens such as influenza and HIV; these antibodies hold promise as biological drugs for passive immunization or treatment (2–5). However, it is not clear that the relatively specific recognition motifs of monoclonal antibodies can accommodate the diverse antigenic landscape in the swarms of viruses present in infected patients or populations. Fortunately, mammalian immune systems fight viral quasispecies with repertoires containing diverse variants of antibodies (6, 7), so the alternative strategy of active vaccination to induce a broad repertoire of potent antiviral antibodies is promising.

Monoclonal antibody studies provide snapshots of the immune response, but what is also needed is a comprehensive method



Deconstructing the recognition pattern of complex mixtures of polyclonal antibodies. Probing the specificities of serum antibodies in HIV-infected subjects for reactivity to particular diverse field strains allows for the identification of the dominant antigenic sites recognized on the gp120/gp41 protective antigens by that individual.

for the study of the global response of the antibody repertoire. Furthermore, the antibodies in serum, which derive principally from long-lived plasma cells in the bone marrow, may differ in specificity from the antibodies encoded in circulating memory B cells that are most often used for monoclonal antibody studies (8). Thus, methods for analyzing the clones in polyclonal serum are needed. The work of Georgiev *et al.* shows that patterns of recognition of complex mixtures of antibodies in human serum can be deconstructed by comparing their reactivity to a diversity of HIV field strains (see the figure). This simple approach could allow us to define the molecular and structural basis for neutralizing polyclonal antibody responses in great detail, thereby facilitating the rapid evaluation of the immunogenicity of experimental HIV vaccines.

Computational methods reveal the nature and diversity of antibodies in HIV-infected individuals.

There are several surprising and informative features of these new results. First, a relatively small panel of viruses (fewer than three dozen) reveals the pattern of antigenic recognition by polyclonal HIV immune serum. This finding provides a new technique for evaluating the quality and breadth of the antibody response to new experimental HIV vaccines, but moreover suggests that the variability in HIV field strains is tractable. Second, the results reveal different patterns of recognition among individuals. This finding is interesting because it has long been a mystery how viruses can use one or a few amino acid changes in a single

epitope to escape the neutralization activity of polyclonal antibody responses directed to a large number of antigenic sites. Influenza virus antigenic drift is a classic example of this phenomenon. The results shown by Georgiev *et al.* with HIV suggest that each individual has a private experience characterized by dominant recognition of one or a few, but not all, major antigenic sites.

If the variability of viral glycoproteins such as the HIV envelope is infinite, then universal immunization against such pathogens will never be possible. Fortunately, viral variability is large but ultimately constrained. The HIV envelope in diverse strains exhibits a large degree of sequence variation and also length polymorphism and differences in predicted N-linked glycosylation sites. However, the scale of information on the observed variability is manageable. With contempo-

rary high-throughput sequence analysis techniques and computational processing, the extent of sequence variability in individuals can be defined with certainty and followed over time (9). Widespread collections of virus field strains from around the world reveal dominant features of the limits of sequence diversity that can be described with phylogenetic concepts such as clades. We now can compare the natural sequence variability in the major structures that induce neutralizing antibodies—regions such as the conserved membrane-proximal external region, the CD4 receptor binding site, and glycans. Georgiev *et al.* show that relatively small panels of HIV field strains can be interrogated for susceptibility of serum samples in bioassays that are amenable to execution on conventional robotic liquid-handling systems.

Antibody repertoires arising in response to viral infections appear to exhibit patterns of emergent behavior characteristic of classical self-organizing systems, arising from relatively straightforward rules of affinity and specificity that determine the fate of individual B cells and thus the antibodies

they secrete. Each B cell must meet its own fate without any apparent mechanism for central coordination of the specificity of the immune response (each clone is an autonomous component of the response that reacts depending on exposure to T cell help, antigen, and the affinity of binding afforded by genetically encoded variable genes). Still, antibody repertoires exhibit complex behavior that solves the difficult geometric problem of dealing with virus swarms by sustaining a large collection of antigen-specific antibodies. The question now is whether we can manipulate patterns of recognition of the human immune response with rational structure-based vaccine design.

The recent revolution in antibody technologies offers new tools that are beginning to provide traction for solving the problem of immunity to antigenically diverse pathogens such as HIV and influenza—perhaps the central challenge of our time in the field of immunology. Vaccinology, until now an empiric science based on inactivating or attenuating organisms or expression of whole wild-type proteins, is undergoing a transi-

tion to a rational process based on large-scale nucleotide and amino acid sequence discovery, structure determination, and antigenicity (10–12). The convergence of increasingly successful structure-based immunogen design (12, 13), with clear definition of the limits of antigenic diversity in the HIV universe, suggests that we should be optimistic about the promise of next-generation HIV vaccine design and testing.

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APPLIED PHYSICS

A Fresh Start for Foam Physics

Denis Weaire

Numerical simulation is making ever greater inroads into the toughest problems of materials science, involving coupled effects on different scales. In the case of foam physics, Saye and Sethian on page 720 of this issue, have set out to meet this challenge with detailed simulations of the gas and liquid dynamics that dictate events in the life of bubbles (1).

The static equilibrium structure of a liquid foam is almost entirely determined by the minimization of its surface energy. The idealized model that neglects everything else might be called the Plateau foam, in honor of the 19th-century physicist who first described conditions for stable foams (2). This model poses intriguing puzzles for geometers, so physicists and mathematicians have found common cause, and even attended common conferences, to debate its deep questions.

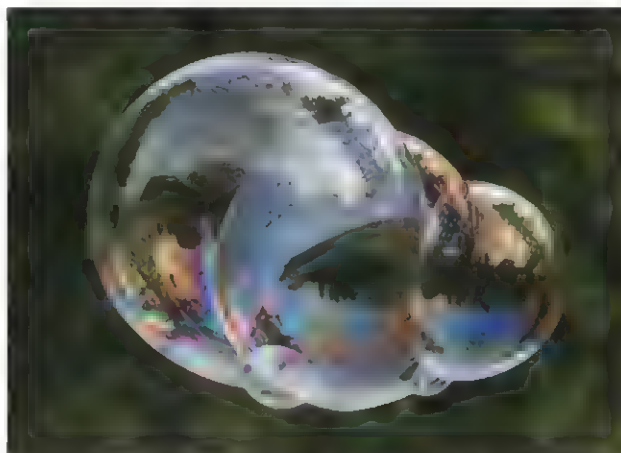
In 1887, the model was deftly applied by Lord Kelvin to calculate the shape of bubbles packed in a particular crystalline arrangement, in the course of his personal, and rather quixotic, quest for the nature of the ether of space (3). However, analytic tricks cannot in general capture the subtle shapes of foam

A numerical simulation moves beyond explaining the static structure of foams and describes their temporal evolution.

cells, even in equilibrium. It was Brakke's Surface Evolver software (4) that opened up the field in modern times. It would be hard to overestimate his remarkable contribution to the study of foams in equilibrium and related research areas.

After more than a century of admiring Plateau foam, and several decades of analyzing its equilibrium structures, it appears to be time to move on to a more general and practical model more capable of describing local dynamics, such as is implicated in the bursting of a bubble and its aftermath. The task is inviting but not easy. To do so, Saye and Sethian have developed a formalism to accurately describe local fluid motion within bubbles, soap films, and their junctions (the so-called Plateau borders).

Three phases of evolution of a small foam sample, such as shown in the figure, are identified and separated for the purposes of simulation. They are the approach to equilibrium, involving rearrange-



Bubble dynamics. There is much to contemplate and simulate in the evolution of a cluster of bubbles. The model of Saye and Sethian can describe some aspects of these dynamical processes.

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ments of bubbles, followed by liquid drainage through the films and Plateau borders, and finally film rupture caused by thinning. This last event throws the system far out of equilibrium, so that we may return to the first phase, and so on.

More approximate or empirical descriptions of this motion in foams near equilibrium have been successful in analyzing many important practical scenarios such as arise in chemical engineering (5). For example, consider the case of local fluid flow when bubbles are rearranged by an imposed shear. In the Plateau foam, these events are considered to be instantaneous and punctuate any slow (i.e., quasistatic) evolution of a structure. In reality, these events occur on a finite time scale that is determined by dissipation associated with fluid viscosity. Time scales lie at the heart of the mysteries of foam rheology (the description of its movement): Foam belongs in the category of a complex fluid.

The new methodology should soon offer fresh insights. Still, there are many real-world cases that cannot be described by the present

model. It is formulated for “dry” foams containing little liquid, as is the case of large bubbles in equilibrium under gravity. Far from equilibrium, for example, in the churning foam of a washing machine, we encounter wet foams that do not get a chance to drain.

Also, a foam out of equilibrium is subject to subtle dynamic effects associated with its surfactant-covered surfaces. Surface energy depends on surfactant surface concentration, which may vary spatially or temporally, and is coupled to bulk concentration. The term “Marangoni effect” is often applied to the phenomena that arise from this dependence, of which the most important is the very existence of the foam in a reasonably stable state (but it may be more familiar as the “tears of wine” effect for a mixed water-alcohol solvent). Surfactant-covered surfaces have a complex rheology of their own, contributing obscurely to that of the foam as a whole and also influencing the internal hydrodynamics of drainage.

Despite the eminent precedents and the help (as well as the criticism) of phys-

ical chemists, physicists have so far failed to grapple effectively with this complex of complications at the film surface. It has proved very difficult to formulate surface effects in a reliable and general way, but this impasse has somehow to be confronted if the new dynamic methodologies are to be fully realistic. However, this new class of numerical modelers has laid foundations for a fresh start. These efforts arrive just in time to help confront the mass of new data soon to be provided by x-ray tomography. It can let us look inside foams and can even be time-resolved so that the dynamics of local structural changes can be revealed.

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PHYSICS

Controlling Atomic Line Shapes

C. D. Lin and Wei-Chun Chu

The spectroscopy of light absorption is an essential tool for uncovering the microscopic structure of a material. The observed spectral line positions reveal the energy levels of the excited quantum states, whereas the line shapes are determined by how the material relaxes after light is absorbed. In the optical frequency regime, the absorption profile has a symmetric shape. By coupling the material to an intense optical laser, however, the absorption can be controlled, leading to many interesting phenomena such as electromagnetically induced transparency (EIT) (1), slow and stopped light (2), and others. Extending such manipulations to extreme ultraviolet (XUV) and soft x-ray frequencies has presented a challenge. With the advent of intense ultrafast few-femtosecond infrared lasers in recent years, as reported on page 716 of this issue, Ott *et al.* (3) demonstrate that such manipulations are now possible.

In the XUV region, the absorption line shape is described by the asymmetric Fano

profile (4). The asymmetry results from the quantum interference of two ionization pathways—one by direct ionization, the other via excitation to an unstable bound state followed by autoionization. In their experiment, Ott *et al.* show that they can change the shape parameter q of a Fano resonance by adjusting the intensity of a coupling laser. This achievement owes much to their high-resolution spectrometers, which can trace accurate line profiles of Fano resonances. Because attosecond XUV pulses are used to excite the atom, the same setup will be able to control the dynamics of a many-electron wave packet, or specifically the reaction dynamics, on attosecond time scales.

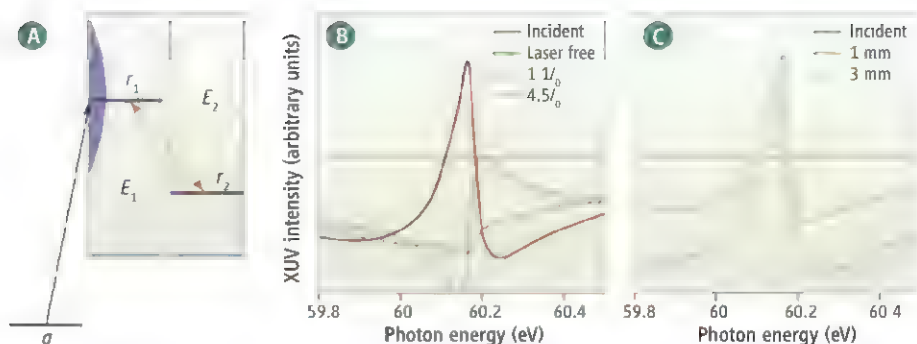
Ott *et al.* co-propagated a broadband XUV attosecond pulse train and a few-cycle near-infrared (NIR) laser with a fixed time delay in a helium target. At a laser intensity of about 2 TW/cm^2 , they found that the asymmetric Fano profiles of the doubly excited states turn into symmetric Lorentzian ones, and the symmetric profiles of the singly excited states, at much lower energies, turn into asymmetric ones. They attribute the change in line shapes to the additional phase acquired by the

Intense infrared lasers can be used to control the spectral line shapes of atoms, with implications for spectroscopy and quantum dynamical processes.

Fano resonances in the presence of the NIR laser. Thus, by tuning the laser intensity, the Fano profiles could be manipulated. Unfortunately, only the data at a fixed time delay were reported; thus, the dynamics of autoionization was left unexplored. The analysis also assumes that the laser field could only change the shape parameter q and that the line shape stays in the general form. This picture also omits the effect of ionization and coupling with other states by the intense laser field. Follow-up experiments will be designed to examine resonance profiles at different time delays and to analyze the data with possible deviations from the Fano line shape.

The same type of experiments using broadband XUV and intense IR pulses with additional control tailored to specific systems should be within reach in the coming years. Nonlinear optics developed with infrared and visible lasers can be readily extended to experiments that use both XUV and IR frequencies (5). In a typical EIT system involving the ground state and two Fano resonances, $2s2p$ and $2s^2$, in the case of helium atom (see the figure, panel A), a 200-attosecond pulse is used to populate $2s2p$ in the presence of a

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Changing shape. (A) Schematics of autoionizing states initiated by an attosecond pulse and coupled by an ultrashort intense laser pulse. The attosecond pulse pumps electrons from the ground state g to the resonance state r_1 and the background continuum E_1 . An intense laser of a few femtoseconds couples r_1 to r_2 , which is embedded in the background E_2 . The decay lifetimes of both resonances are long relative to the pulse durations. The purple curve indicates that the bandwidth of the attosecond pulse covers the whole resonance line shape near r_1 . (B) Light transmission spectra of a 200-attosecond pulse near the 2s2p resonance through a 2-mm helium gas sample at different coupling laser intensities. In the laser-free condition, the original Fano line shape is detected with a positive q . At the peak intensity of $1.1 I_0$ ($I_0 = 1 \text{ TW/cm}^2$), the resonance disappears where only the background remains. At the peak intensity of $4.5 I_0$, the q parameter changes sign. The part of the spectrum higher than the incident light represents emission. (C) Same as (B) but for a fixed coupling laser intensity of $4.5 I_0$ and for different propagation lengths. At 1 mm, the resonance part is enhanced while the background attenuates. This enhancement persists along the propagation while the background keeps dropping, as shown by the 3-mm curve. Thus, a broadband XUV pulse can be shaped when propagating through a laser-dressed helium gas medium.

few-femtosecond 540-nm laser with adjustable time delays and intensities (around 1 TW/cm^2). The laser resonantly couples the two autoionizing states in order to induce the Rabi oscillation between the two states. This coupling introduces a new pathway for the autoionization of 2s2p—via $2s2p \rightarrow 2s^2 \rightarrow 2s2p$ —in addition to the direct one. The interference between the two pathways is adjustable by the laser intensity and the time

delay. The transmission of the XUV light near the 2s2p resonance is shown in the presence of the overlapping laser pulse (see the figure, panel B). At the lower laser intensity, the resulting flat transmission curve indicates that the absorption by the 2s2p state vanishes as it is transferred to $2s^2$. At higher laser intensity, the originally positive q parameter becomes negative and a strong emission peak appears. This is an example of lasing without

population inversion. If a helium gas medium is dressed by such a laser pulse, then the XUV would emerge from the medium with a narrow bandwidth of about 50 meV (see the figure, panel C), illustrating the shaping of XUV pulses via a laser-dressed Fano resonance.

For decades, it has been shown that the optical properties of a material medium can be dramatically modified by controlling the quantum states of an optical electron with lasers. The experiment of Ott *et al.* demonstrates that such control can now be extended to inner-shell electrons that are accessed via attosecond XUV or soft x-rays. This experiment also shows that the control is to be carried out by tuning the time delay between the XUV and the IR laser. As attosecond XUV and soft x-rays are becoming readily available (via high-order harmonic generation in a gas medium) in many ultrafast-laser laboratories, we can anticipate that coherent control of quantum states of inner-shell electrons—not only for atoms and molecules, but also for nanostructures and other materials—will become widely feasible soon. With such a high degree of control between light and matter emerging, what one can achieve in the future will be limited only by our imagination.

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NEUROSCIENCE

Why Adults Need New Brain Cells

Olaf Bergmann and Jonas Frisén

Few new cells are generated in the adult brain and spinal cord, and as such, nervous system plasticity was long thought to only involve modulating the contacts between preexisting “old” neurons. That view is changing. New neurons, as well as glial cells (specialized supporting cells), in the adult brain do indeed mediate certain types of plasticity, and the malfunction of such processes may cause neurological or psychiatric disease. On page 756 of this issue, Freund *et al.* (1) report a link between

cognitive challenges, adult brain neurogenesis, and the development of individuality. This relationship supports the idea that a key function of adult neurogenesis is to shape neuronal connectivity in the brain according to individual needs.

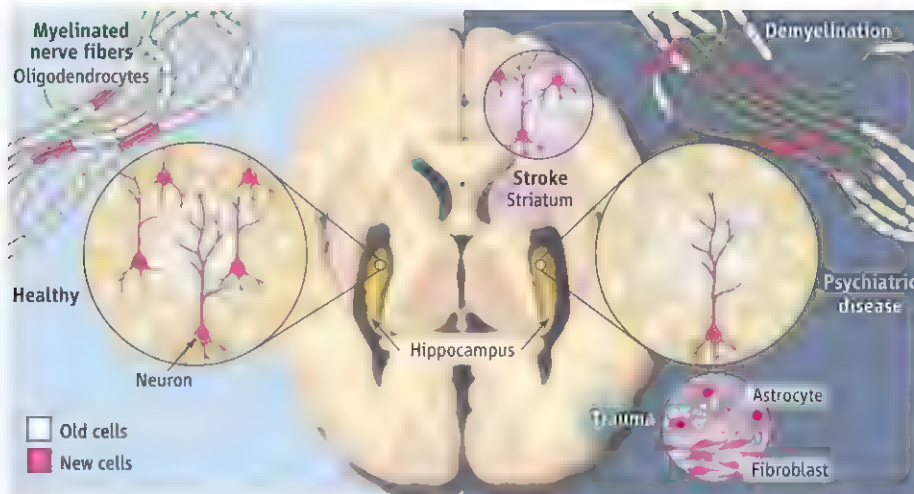
Neurons are generated until shortly after the time of birth, with the exception of two small areas in the brain of most mammals—the olfactory bulb and hippocampus, where neurons are added by neural stem cells throughout life (2, 3). Humans, however, appear unique in that there is no detectable olfactory bulb neurogenesis (4, 5). Newborn neurons have special electrophysiological features for about 1 month after their genera-

Neurogenesis and gliogenesis shape connectivity in the adult brain, influencing plasticity and repair.

tion, after which they become indistinguishable from the older neurons (2, 3). The continuous production of new neurons may serve to maintain a pool of neurons with such special properties. Adult neurogenesis has a specific function in discriminating similar experiences, a process called pattern separation. Newborn hippocampal neurons help separate the perception of similar events for storage as distinct memories—for example, remembering not only that you parked your car in the parking lot, but also where in the parking lot (2, 6). Pattern separation is critical for adapting to a complex environment.

How adult neurogenesis relates to brain plasticity in complex environments has been

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New cells in old brains. In the healthy human brain (left), new neurons are added in the hippocampus, and new oligodendrocytes form myelin sheets to insulate nerve fibers. The generation of neurons and nonneuronal cells may change in pathological conditions (right).

difficult to address. Freund *et al.* monitored the exploratory behavior of a large cohort of genetically identical mice in a complex environment for 3 months. Variation in individual behavior increased over time, just as human monozygotic twins become more different with age and develop increasing individuality. Physical activity promotes adult neurogenesis and, as expected, the animals that moved longer distances had more new neurons. However, the authors found that the size of the area that the animals regularly explored had an even stronger influence on hippocampal neurogenesis. The animals that regularly roamed over large areas were consequently exposed to more cognitive challenges, and had the most new neurons. The study connects structural and functional brain plasticity in a more natural environment than previously tested and shows how the brain is sculpted by interaction with the environment. How cognitive challenges impinge on the epigenetic regulation of neurogenesis will be an interesting topic for future studies.

What happens if adult neurogenesis fails? Altered adult hippocampal neurogenesis has been implicated in psychiatric disease (see the figure). Fewer new neurons, which impair pattern separation, may, for example, underlie difficulties in distinguishing threats from similar, but safe, situations, thereby contributing to a generalized perception of fear and anxiety in posttraumatic stress disorder (6). Most antidepressant treatments promote hippocampal neurogenesis, and some of the effects of antidepressants in animal models are indeed dependent on increased neurogenesis (7).

The generation of glial cells is much more widespread than neurogenesis in the

adult brain, and there is increasing evidence that they also influence neural plasticity. Oligodendrocytes (a type of glial cell) wrap their cell membranes around nerve fibers to form insulation called myelin, which vastly increases nerve fiber conduction velocity and the speed of neural processing. Myelination is a largely postnatal process, which is believed to underlie some of the functional maturation of the brain into early adulthood. Myelination is dynamically regulated, and practicing a skill like juggling balls results in increased volume of myelinated nerve fiber tracts and presumably improved performance of the neural circuitry involved in carrying out the task (8). It is thought that mature oligodendrocytes cannot modulate their myelination, but that new myelin requires the generation of new oligodendrocytes (9). Oligodendrocytes are produced by dedicated progenitor cells (10), which account for most of the cell proliferation in the adult central nervous system.

Oligodendrocytes and myelin are lost in primary demyelinating diseases such as multiple sclerosis, as well as secondarily in many neurological disorders, resulting in impaired nerve fiber conductance. Myelin can be regenerated by new oligodendrocytes to restore neurological function (10). However, the regeneration is often incomplete and a neuron eventually dies if its denuded nerve fiber is not remyelinated, resulting in a permanent neurological deficit, as often seen in, for example, advanced multiple sclerosis. There is very limited generation of astrocytes, another major class of supporting cells, in the mature nervous system. After most types of injuries, however, there is a large local increase in the number of astrocytes, gener-

ated by neural stem cells and/or by duplication of preexisting astrocytes. Astrocytes, together with pericyte-derived connective tissue cells, form permanent scar tissue (11, 12), which is thought to inhibit the regrowth of neuronal processes (axons) (13, 14).

The realization that new neurons and glial cells have substantial influence on neural plasticity has changed our view of the adult brain and point to new potential targets for therapeutic intervention. Demyelination and scarring are common to many neurological conditions, and as gliogenesis is not locally restricted, finding ways to modulate it can have broad implications for treating neurological disease. Neurogenesis, by contrast, is under strong local control, and it is difficult to envisage replacing damaged neurons that affect nonneurogenic parts of the brain or that affect the brain broadly. One exception is stroke, which triggers the generation of neurons in the striatum, a region where neurons normally are not added postnatally. The new neurons may mediate some of the spontaneous functional improvement that often is seen (15).

That neurogenesis normally appears restricted to the hippocampus in humans makes psychiatric disease and cognitive impairment the most obvious targets for intervention. It may seem farfetched to develop pharmaceuticals that trigger the production of new nerve cells, but some of the most commonly prescribed drugs today—antidepressants—have this effect. However, none of them were developed with the aim to promote neurogenesis, and molecular understanding of neurogenesis will hopefully aid in the rational development of new classes of drugs for psychiatric disease. Moreover, understanding how adult neurogenesis influences brain plasticity may teach us, as Freund *et al.* explain, how living our lives makes us who we are.

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Bacterial Subversion of Host Innate Immune Pathways

Leigh A. Baxt,^{1*} Anna Cristina Garza-Mayers,^{2*} Marcia B. Goldberg^{1,2†}

The pathogenesis of infection is a continuously evolving battle between the human host and the infecting microbe. The past decade has brought a burst of insights into the molecular mechanisms of innate immune responses to bacterial pathogens. In parallel, multiple specific mechanisms by which microorganisms subvert these host responses have been uncovered. This Review highlights recently characterized mechanisms by which bacterial pathogens avoid killing by innate host responses, including autophagy pathways and a proinflammatory cytokine transcriptional response, and by the manipulation of vesicular trafficking to avoid the toxicity of lysosomal enzymes.

In recent years, cellular mechanisms involved in membrane trafficking and the elimination of intracellular particles—including pathogens, membrane fragments, and protein aggregates—have been elucidated in increasing molecular detail. Simultaneously, a variety of cellular mechanisms for sensing infecting pathogens have been discovered (*1*), including recognition of pathogen-associated molecular patterns (PAMPs) and host-derived danger signals induced by microbes. These mechanisms variously result in engulfment and elimination of the pathogen via autophagy, proinflammatory cytokine production, lytic cell death via pyroptosis, and induction of inflammatory responses in the host organism.

These innate immune mechanisms work in parallel with one another and in a coordinated fashion to eliminate bacterial threats. Each provides specialized protection for specific subcellular compartments (*1*). Which mechanism is most effective or appropriate to defend against a particular pathogen depends largely on the subcellular compartment occupied by the pathogen (Fig. 1).

In parallel with the explosion of insights into fundamental innate immune processes, specific processes by which pathogenic microorganisms subvert these innate immune pathways have been discovered. Prototypical bacterial mechanisms for altering host innate immunity are the specialized secretion systems (particularly types III and IV and, in some instances, type VI) of Gram-negative bacteria, which enable infecting bacteria to inject effector proteins directly into the cytoplasm of host cells. The effector proteins directly modify the function of host factors engaged in innate immune signaling, cytoskeletal dynamics, membrane trafficking, phosphoinositide lipid metabolism, host cell signaling, ubiquitin modification pathways, transcription, and protein modification, among others. In addition, the host

innate immune response has evolved the capability to recognize these secretion systems.

In this Review, we examine examples from a range of mechanisms by which bacterial patho-

gens avoid killing by host-cell innate responses. In turn, the discovery of these mechanisms of pathogen survival has contributed to our understanding of the underlying protective host pathways.

Evading Autophagy

One of the earliest defense responses encountered by bacterial pathogens within minutes or hours of entry into a host cell is autophagy, a process that engulfs and delivers intracellular bacteria for lysosomal degradation. Several pathogens have evolved mechanisms to evade the autophagic response, but we have mechanistic insight into only a few. Inhibition of autophagy is mediated by secreted bacterial proteins that block various steps of the process.

Evasion of Autophagic Recognition

We know that cytoplasmic bacterial pathogens circumvent recognition by the autophagy machinery. For example, in *Shigella*, evasion is by direct inhibition of autophagic proteins, whereas

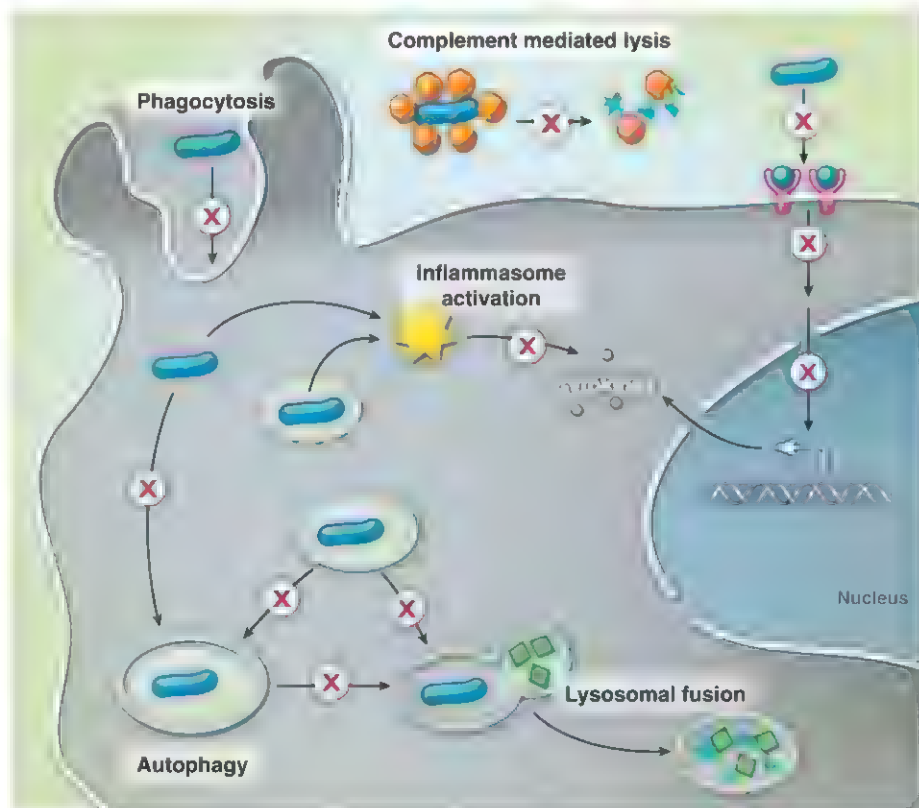


Fig. 1. Innate immune responses commonly encountered by pathogenic bacteria. The specific immune responses faced by bacteria during infection are dictated in part by the subcellular compartment that they occupy. Extracellular bacteria are subject to phagocytosis by professional phagocytes and to complement-mediated lysis. Intravacuolar and intracytoplasmic bacteria are subject to engulfment by autophagy and killing via lysosomal fusion with the membrane-bound compartment. Detection of bacterial PAMPs by extracellular or intracellular receptors activates signaling cascades that lead to a proinflammatory transcriptional response. Detection of bacterial PAMPs in the cytosol also triggers activation of inflammasomes, which activate proinflammatory cytokines. Steps in these pathways known to be inhibited by bacteria are marked with a red X. Blue ellipses and circles, rod-shaped and coccoid bacteria, respectively; orange hexagons, complement; green diamonds, degradative enzymes within a lysosome; yellow star, inflammasome; pink "cups" on cell surface, Toll-like receptors (extracellular PRRs); white arrow, proinflammatory cytokine transcriptional response.

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Listeria monocytogenes recruits host cell proteins that mask the pathogen from recognition (2, 3). Both of these pathogens induce their uptake into mammalian cells: *Shigella* predominantly into epithelial cells of the colon and *L. monocytogenes* predominantly into macrophages.

Shortly after entry, *Shigella* escapes the uptake vacuole and, mediated by the surface protein IcsA, polymerizes host actin at the bacterial surface into a propulsive tail that facilitates bacterial spread. In addition to recruiting host actin polymerization machinery, IcsA is recognized by the host's autophagy protein Atg5 (2). *Shigella* counters this attack by means of a protein secreted by the organism's type III secretion system, IcsB, which binds IcsA to block Atg5 recognition, thereby protecting the bacterium from autophagy. The *Burkholderia pseudomallei* type III-secreted effector protein BopA is a homolog of IcsB and is likewise required for evasion of autophagy, although whether a similar mechanism operates is unknown (4).

Like *Shigella* spp., *L. monocytogenes* escapes the uptake vacuole and spreads by polymerization of an actin tail. Actin-tail formation by *L. monocytogenes* is also mediated by a surface protein, ActA, which is functionally distinct from *Shigella* IcsA. *L. monocytogenes* uses two mechanisms for disguise, both requiring host proteins. First, ActA recruits the host actin polymerization machinery, consisting of Arp2/3, actin, and Ena/VASP proteins, which polymerize and elongate actin tails at the bacterial surface. In the absence of ActA, significantly more *L. monocytogenes* are engulfed in autophagosomes (3). The combination of recruitment of host actin-associated pro-

teins and the formation of a propulsive actin tail allows *L. monocytogenes* to evade autophagic recognition. A second *L. monocytogenes* protein, internalin K (InlK), has a nonredundant role in avoidance of autophagy, such that if InlK and ActA are lacking, more bacteria are targeted to autophagy than if one or the other is missing (5). Recruitment of the host's major vault protein to the bacterial surface by InlK correlates with escape from autophagosomes, albeit by an unknown mechanism (5).

Inhibition of Autophagy

After pathogen recognition, the autophagy initiation complex assembles. This complex comprises combinations of Unc-51-like kinase, Beclin-1 (Atg6/Bcl-2-interacting protein 1), phosphatidylinositol 3-kinase, Vps15, Vps34, and Atg14. During assembly a double-membrane structure (phagophore) forms that elongates into a mature autophagic vacuole that encloses the desired target for destruction.

The membranes of the autophagosome become decorated with the lipidated phosphatidylethanolamine (PE)-conjugated form of LC3 (LC3-II). This maturation process appears to be disrupted by several pathogens, resulting in a decrease of LC3-II relative to uninfected cells (6, 7). For example, *L. pneumophila* creates an endoplasmic reticulum (ER)-derived vacuolar niche, the *Legionella*-containing vacuole (LCV), which is protected from innate immune responses of the host. RavZ, a *L. pneumophila* effector protein, secreted by the specialized type IV secretion system, protects intracellular organisms from autophagosome maturation by irreversibly deconjugating

PE from LC3 (7). Such deconjugation involves proteolytic cleavage of the lipid moiety one residue amino-terminal to the point of conjugation, which prevents re-conjugation (7). However, in the absence of RavZ, many LCVs survive, indicating that other mechanisms of autophagy evasion function during infection.

Modulation of Autophagosomal Maturation

Bacterial pathogens replicating within vacuoles in host cells can still be recognized and targeted for autophagy by the host. Consequently, several bacterial pathogens have evolved mechanisms to modulate autophagosomal maturation, apparently by inhibition of autophagosomal-lysosomal fusion. Mechanistic insight into these processes is largely lacking, but preliminary studies with several pathogens suggest that secreted bacterial effectors are involved (Fig. 2). For example, the Gram-negative obligate intracellular pathogen *Anaplasma phagocytophilum* lives within an autophagosome-like compartment that consists of a double lipid bilayer decorated with several autophagosome markers. The Ats-1 protein is secreted by the organism's type IV secretion system and appears to induce autophagy through binding the initiation complex component Beclin-1 (8). Despite robust induction of autophagy, vacuoles containing bacteria lack lysosomal markers, indicating that autophagosomal-lysosomal fusion is inhibited.

Do Bacterial Pathogens Modify the Overall State of Autophagy?

In addition to removing invading pathogens, autophagy pathways recognize other factors that accompany infection. Infection-specific intracyto-

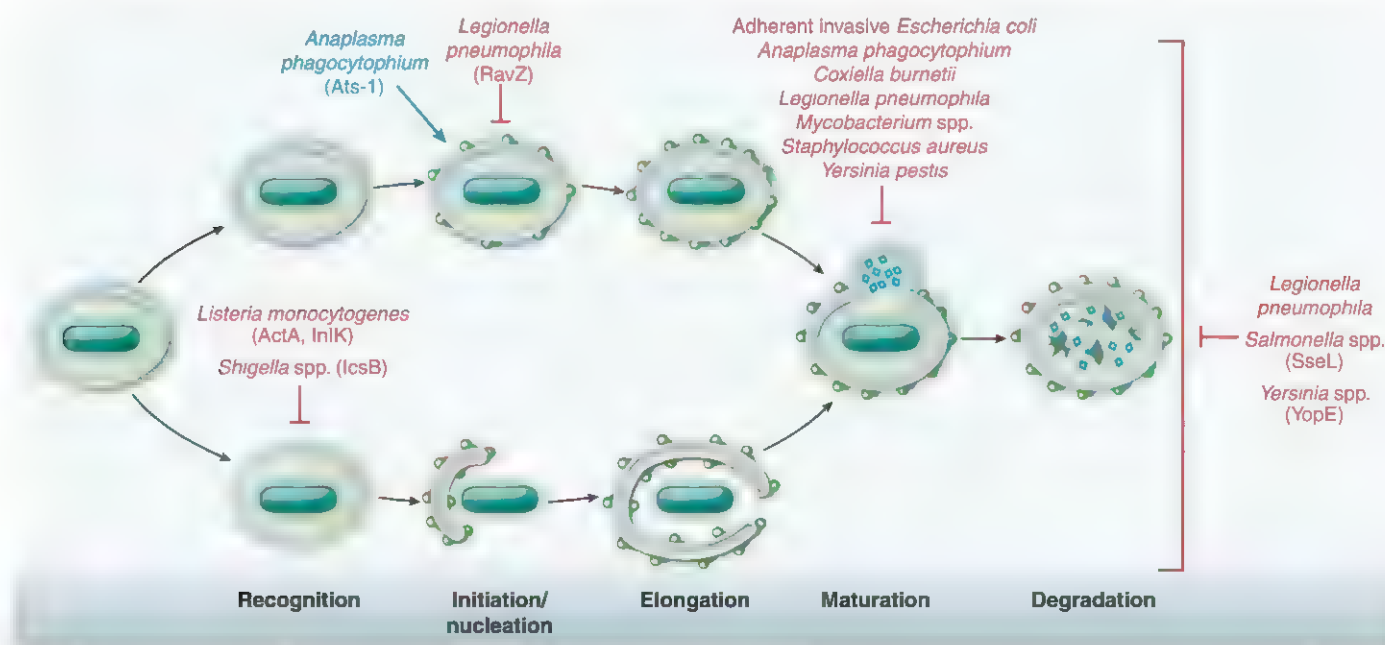


Fig. 2. Manipulation of autophagy by intracellular bacteria. Autophagy pathways and sites at which bacterial effector proteins interfere with these pathways are depicted for intravacuolar (top) and intracytosolic (bottom) pathogens. Multiple bacterial effector proteins from a wide range of bacteria have been identified as interacting with

these pathways (2, 3, 5–10, 39–43, 44). Bacterial strains are given, and the relevant effector proteins are indicated in parentheses. Blue arrow, activation of cellular process; black arrows, progression in autophagy pathways; red lines, inhibition of cellular process; green circles, foci of LC3-II; compartment containing blue diamonds, lysosome.

plasmic aggregates, or aggresome-like structures, are of particular interest. Aggresomes are ubiquitinated by host ligases, which enables them to be recognized by the autophagy pathway. This leads to activation of autophagy in the cell, thereby increasing the overall level of autophagy and increasing the likelihood of pathogen destruction. Ubiquitinated intracytoplasmic aggregates have been observed to accumulate around *Salmonella*-containing vacuoles (SCVs), but this pathogen circumvents any harm to itself by means of a deubiquitinase, SseL, secreted by the organism's type III secretion system (9). Similar aggregates form during infection of macrophages by *L. pneumophila*, and here expression of the type IV Dot/Icm specialized secretion system blocks their ubiquitination (10). The genomes of several pathogenic *Escherichia coli* encode proteins with considerable sequence similarity to SseL, including conservation of an active cysteine residue, leading us to speculate that this mechanism of autophagy suppression may be widespread among bacterial pathogens and raising the possibility that damping the overall level of autophagy may also be of benefit to extracellular pathogens.

Interfering with Induction of the Proinflammatory Transcriptional Response

In response to microbial infection, a proinflammatory transcriptional response is activated that

triggers recruitment of phagocytic cells and other components of the immune response to the site of infection. Activation is mediated by mitogen-activated protein kinase (MAPK) cascades and translocation of nuclear factor κ B (NF- κ B) into the nucleus, leading to increased transcription of a panel of immune molecules, including the proinflammatory cytokines interleukin-1 β (IL-1 β) and IL-18. The effector activators of these pathways are the pattern-recognition receptors (PRRs), which recognize microbial PAMPs in the extracellular, vacuolar, and cytoplasmic compartments. Effector proteins from bacterial pathogens intercept NF- κ B activation and MAPK kinase (MAPKK) signaling in numerous ways (Fig. 3), in several cases via previously undescribed enzymatic or molecular activities.

The *Shigella* spp. IpaH family of type III secreted effector proteins function as E3 ligases, one of which modulates NF- κ B activity (11). Similarly, Cif (a type III secreted effector of pathogenic *E. coli*) and Cif homolog in *Burkholderia pseudomallei* (CHBP) block degradation of the inhibitor of NF- κ B (I κ B α) by deaminating a glutamine residue of the ubiquitin-like molecule (Ubl) NEDD8 (neural precursor cell expressed, developmentally down-regulated 8) and, to a lesser extent, ubiquitin itself (12).

OspF, a type-III secreted effector protein of *Shigella* spp., is a phosphothreonine lyase that

specifically inactivates MAPKs by irreversibly removing phosphate groups (13), reducing the influx of acute inflammatory cells into *Shigella*-infected tissues in mice (14, 15). Similarly, the *Salmonella* spp. homolog of OspF, SpvC, is a phosphothreonine lyase that inactivates phospho-Erk and inhibits inflammation in the intestines of mice (16, 17).

Yersinia spp. translocate into cells the acetyltransferase YopJ (YopP in *Y. enterocolitica*), which inactivates multiple cellular kinases by acetylation of serine and threonine residues (18, 19). YopJ inactivation of MAPKK and inhibitor of NF- κ B kinase subunit beta (IKK β) induces apoptosis, release of activated caspase-1, and secretion of IL-1 β . Inactivation of kinases downstream of the intracellular nucleotide-binding oligomerization domain (NOD)-like receptor (NLR)-2 has a similar effect and, in mice, causes intestinal barrier dysfunction (18–21).

Yet, *Yersinia* spp. also block activation of proinflammatory cytokines. The inflammasome is a pro-caspase-1 containing macromolecular platform containing NLR family proteins in response to PAMPs. Activation results in cleavage of pro-caspase-1 to caspase-1, which processes pro-IL-1 β and pro-IL-18 into their mature and active forms (Fig. 3), leading to both release of the proinflammatory cytokines and pyroptotic cell death. The *Yersinia* type III secreted effector protein

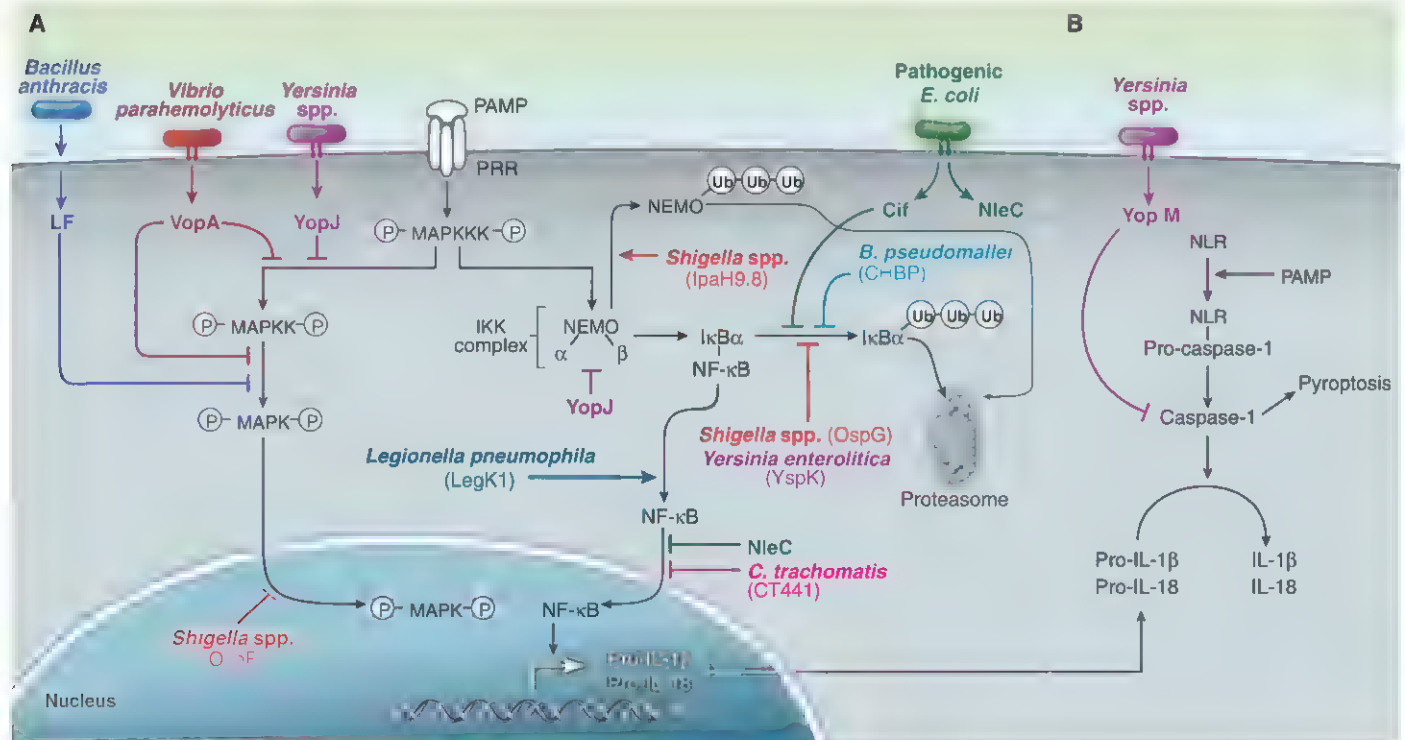


Fig. 3. Manipulation of the proinflammatory transcriptional response by bacterial effector proteins. (A) Pathways of MAPK and NF- κ B activation and sites at which bacterial effector proteins interfere with these pathways are depicted. MAP kinase signaling is triggered by detection of microbial PAMPs by PRRs. Signaling leads to transcriptional activation of proinflammatory responses, including transcription of the cytokines IL-1 β and IL-18. Bacterial effector proteins that have been identified as interacting with these pathways are indicated (11–15, 18, 19, 45–50).

Arrows indicate activation of cellular process; lines denote inhibition of cellular process. MAPKKK, MAPKK kinase; Ub, ubiquitin; LF, lethal factor; P, phosphate. (B) Detection of bacterial PAMPs in the cytosol by NLRs activates the inflammasome pathway. Inflammasome activation leads to processing of caspase-1 to its active form. In turn, active caspase-1 processes the cytokines IL-1 β and IL-18 to their active forms. *Yersinia* spp. YopM interferes with bacterium-induced activation of inflammasomes by binding to mature caspase-1 (22).

YopM binds and sequesters mature caspase-1, preventing substrate binding (22). A four-residue sequence in an exposed loop of YopM, similar to the binding loops in caspase-1 substrates and regulators, is required for YopM-mediated inhibition of caspase-1 (22), indicating that inhibition of caspase-1 occurs by substrate mimicry. Thus, *Yersinia* spp. both block and activate proinflammatory signals. Precisely how these seemingly paradoxical effects of *Yersinia* effector proteins are orchestrated during infection in vivo remains unclear.

Multiple bacterial factors trigger inflammasome activation (23), and inhibition of caspase-1 activation or reduction in release of IL-1 β has been described for many pathogens, although the mechanistic details have been elucidated in only a few instances.

Manipulating Vesicular Trafficking

As well as evading autophagy and intracellular inflammatory processes, survival of bacterial pathogens also requires modification of normal cell functions that contribute to host defense, such as host phagocytic, transport, and secretory pathways. Pathogens enhance their survival by avoiding phagocytosis or subsequent fusion of bacteria-containing vacuoles with lysosomes. For example, it has long been known that *Neisseria gonorrhoeae* inactivates complement using the host's own complement-inhibitory proteins, thwarting complement-mediated opsonophagocytosis and killing (Fig. 1).

Pathogenic *E. coli*, on the other hand, use the type III secreted effector EspF to avoid antibody-independent direct uptake by professional phagocytic cells. A second *E. coli* type III secreted effector, EspJ, inhibits macrophage opsonophagocytosis generally (24).

Modification of Intravacuolar Niches

Another common theme in the promotion of pathogen survival is the exploitation of host guanosine triphosphatase (GTPase) signaling to co-opt host cell vesicle trafficking (Fig. 4). These small regulatory switch molecules cycle between an inactive guanosine diphosphate (GDP)-bound form and an active GTP-bound form with the help of guanine nucleotide exchange factors and GTPase-activating proteins.

The *S. Typhimurium* type III secreted effector SopB, a phosphoinositide phosphatase, is required for the generation and persistence of phosphatidylinositol 3-phosphate on SCVs and for SCV maturation (25). SopB's phosphatase activity alters the electrostatic membrane surface charge of the SCV, affecting the recruitment of Rab GTPases to the SCV and, ultimately, inhibiting SCV fusion with the lysosome (26).

Additionally, the *S. Typhimurium* type III secreted effector, SifA, promotes the formation of *Salmonella*-induced filaments (SIFs), membranous tubules that extend from the SCV to the plasma membrane. SIFs and other types of tubules induced during *Salmonella* infection are thought to

be required for vacuole stability. SifA also sequesters the small GTPase Rab9 and other host proteins to subvert retrograde trafficking of mannose 6-phosphate receptors, thereby decreasing lysosomal activity in infected cells to favor intracellular growth (27). Thus, *S. Typhimurium* can modify Rab GTPase activity to both exploit vesicular trafficking and maintain the integrity of its own vacuolar niche.

During *L. pneumophila* infection, bacterial induction of LCV formation is critical to its protection from innate immune responses. The *L. pneumophila* protein RalF, which is secreted by the Dot/Icm type IV secretion system, recruits and activates host GTPase adenosine diphosphate ribosylation factor 1 to LCVs for LCV biogenesis (28, 29). *L. pneumophila* also recruits and activates the cellular GTPase Rab1 to the LCV via the type IV secreted effector protein SidM (DrrA) that, along with a second *L. pneumophila* effector (LidA), recruits ER-derived vesicles to the LCV (30). SidM also functions as a GDP dissociation inhibitor displacement factor (31). The type IV secreted effector, LepB, deactivates Rab1 once LCV maturation and fusion with the host ER is complete. Thus, *L. pneumophila* orchestrates the recruitment and activation of Rab1, as well as its timely deactivation and removal, for the maintenance of its intracellular niche.

Although less well understood, *Coxiella burnetii* provides perhaps the ultimate example of vacuolar niche development as a mechanism of

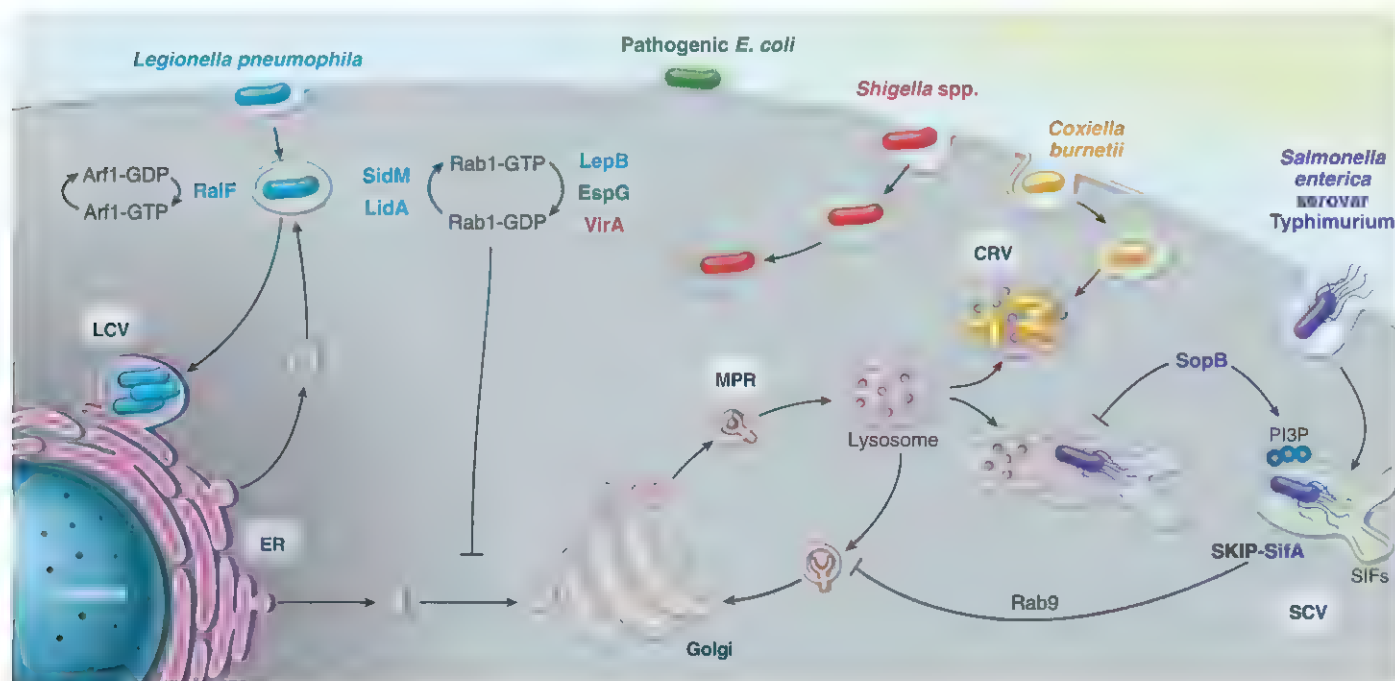


Fig. 4. Host GTPase signaling and vesicular trafficking by pathogenic bacteria. Cellular vesicle transport and secretory pathways contribute to host defense by containing pathogens in vacuoles. In phagocytic cells, lysosome fusion with the phagosome leads to killing of intraphagosomal bacteria by lysosomal enzymes. In many cell types, bacterial manipulation of vesicular trafficking promotes bacterial survival. This occurs largely by the manipulation

of GTPase signaling by bacterial effector proteins. The examples of bacterial manipulation of host secretory and transport pathways discussed in the text are shown here (25–32, 35, 36), and steps at which secreted bacterial effector proteins intersect host vesicular trafficking are indicated. CRV, *Coxiella*-replicative vacuole; MPR, mannose-6-phosphate receptor; PI3P, phosphatidylinositol 3-phosphate.

immune evasion in that it survives in a lysosome-like environment. The *Coxiella*-replicative vacuole is an acidic autophagolysosome-like vacuole generated by fusion and interaction with the phagocytic, endocytic, and secretory pathways (32). As for other intracellular pathogens, the mechanisms of this distinct adaptation require secretion of effector proteins by a specialized secretion system (33, 34).

Modulating Vesicular Trafficking

The type III secreted effector, EspG, of pathogenic *E. coli* displays GTPase-activating activity for Rab1 that disrupts ER-to-Golgi trafficking and general secretory pathways (35, 36). Interestingly, the EspG homolog, VirA, of *Shigella* spp., displays similar activity to disrupt ER-to-Golgi trafficking and suppress host autophagy (35). Whether EspG alters host autophagy during *E. coli* infection or whether VirA affects the general secretory pathway during *Shigella* infection is not yet clear.

Outlook

The pathogenesis of infection is a constantly evolving battle between the host, which needs to restrict an infecting microorganism, and the pathogen, which needs to replicate and survive for transmission to other hosts. Although the past decade has brought numerous insights into the molecular mechanisms involved in this exchange, the understanding of bacterial resistance to the host response lags behind insights into the responses themselves.

An important reason for this is that most mechanisms of bacterial interference with host innate immune responses have been worked out

in cell culture. In vitro systems oversimplify innate responses due to lack of normal cytokine context, absence of complex tissue and cell types, and use of immortalized cell lines that do not reproduce responses seen in primary cells. Moreover, it is becoming increasingly clear that each host response pathway likely regulates others: For example, autophagy negatively regulates activation of inflammasomes (37, 38), and vesicular trafficking is linked to autophagy (35). Moving forward, studies in intact animals are critically important, as they will allow analysis of the host response under conditions that provide for the interplay of multiple innate immune pathways.

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Cellular Self-Defense: How Cell-Autonomous Immunity Protects Against Pathogens

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Our prevailing view of vertebrate host defense is strongly shaped by the notion of a specialized set of immune cells as sole guardians of antimicrobial resistance. Yet this view greatly underestimates a capacity for most cell lineages—the majority of which fall outside the traditional province of the immune system—to defend themselves against infection. This ancient and ubiquitous form of host protection is termed cell-autonomous immunity and operates across all three domains of life. Here, we discuss the organizing principles that govern cellular self-defense and how intracellular compartmentalization has shaped its activities to provide effective protection against a wide variety of microbial pathogens.

The cell is an outstandingly attractive nutrient source for potential parasites and pathogens. All organisms must therefore have developed the ability to defend against such threats early during evolution (1). In metazoans, specialized immune cells stimulate innate immunity and, in vertebrates, adaptive immunity; how-

ever, most other species rely entirely on intrinsic self-defense for protection, which in some cases has reached an astonishing level of complexity. In archaea and bacteria, for example, even adaptive forms of resistance—long considered the hallmark of vertebrates—contribute to cell-autonomous immunity, as exemplified by the

clustered regularly interspaced short palindromic repeats (CRISPR) system, which recognizes foreign DNA in a sequence-specific manner (2). In metazoans, cellular self-defense synergizes with the whole-body protection provided by traditional immunity to confer pathogen resistance. Here, professional immune cells patrol their environment in search of pathogens, whereas cell-autonomous immunity guards both individual immune and non-immune cells against the immediate threat of infection (3–6). Cellular self-defense thus has the potential to confer antimicrobial protection on most, if not all, cells.

Cell-autonomous effector mechanisms appear conserved across phyla. For example, nitric oxide synthases (NOSs) defend Gram-positive bacteria against other bacilli that share the same environment (7). Epithelial cells in the osmoregulatory

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Malpighian (renal) tubules of flies express NOS to cell-autonomously combat bacteria (8). Similar pathways exist in nonimmune cells of humans and mice (5). Note, however, that these ancient systems have not been inherited unchanged by higher eukaryotes. Viral restriction factors such as the vertebrate tripartite motif (TRIM) protein family or immunity-related guanosine triphosphatases (GTPases) (IRGs) that protect against intracellular bacteria and protozoa are encoded by some of the fastest evolving genes in all of metazoan biology (4, 5). Here, we outline the basic principles of cellular self-defense in animals, with an emphasis on how cells take advantage of their compartmentalized nature and how the defined composition of compartments, as well as the borders between them, are used to antagonize the invasion, replication, and spread of intracellular pathogens.

A Topological View of Cellular Self-Defense

Surveying the entire cell body for the presence of pathogens is a daunting task, particularly for eukaryotic cells whose volume greatly exceeds that of their potential foe (5). The division of eukaryotic cells into membrane-bound compartments poses an additional problem because compartments can provide excellent sanctuaries for pathogens (5, 9). Immune sensors must therefore exhibit a high degree of sensitivity; be targeted to the appropriate location; or be part of a widespread, multi-layered surveillance system to detect infection.

Although compartmentalization provides potential pathogen habitats, it also facilitates unique defense strategies (Fig. 1). Pathogens entering their preferred intracellular niche must cross at least one, and often two or three, physical barriers (cellular membranes). Traversing each barrier requires specific adaptations, and at each step, pattern recognition receptors (PRRs) and other sensory machinery are positioned to alert the host cell to the presence of infection, while restriction factors are poised to inhibit replication (5, 10, 11). Compartmentalization has driven the evolution of compartment-specific sensory receptors capable of detecting pathogen-associated molecular patterns (PAMPs) (12, 13) and danger-associated molecular patterns (DAMPs) (14). Compartmentalization also allows the cell to control the topological distribution of molecules that are either harmful or desirable to the pathogen. This principle has enabled the evolution of powerful antimicrobial effector mechanisms that, although detrimental for the cell in one compartment, are innocuous in others. Finally, compartmentalization enables steep concentration gradients across membranes. These gradients permit cells to survey the integrity of their compartments based on danger receptors that detect the entry of molecules that are normally excluded from a given compartment.

Compartment Borders Restrict Pathogen Movement

Self-defense begins even before pathogens come into contact with the cell surface. Chemorepel-

lents are used to deter microbes from approaching host cells, such as hydrogen peroxide (H_2O_2) generated by the apically localized DUOX2 enzyme (15). Specialized gut epithelial Paneth cells secrete the antimicrobial lectin, RegIIIy, into the lumen of the small intestine to maintain a ~50- μ m germ-free barrier (16). Lack of this sugar-binding protein in mice enables rapid microbial colonization of the intestinal mucosa.

All cells are surrounded by outer membranes, and eukaryotic cells are divided by endomembranes into multiple internal compartments. These membranes constitute physical barriers to infection. Pathogens have evolved different solutions to this challenge. Certain enveloped viruses, such as HIV, herpes simplex virus (HSV), and Rous sarcoma virus, fuse with the plasma membrane to deliver their genome into the cytosol. This fusion event is sensed by the cell using polytopic membrane proteins like STING to trigger antiviral defenses (17). Antimicrobial proteins embedded within the plasma membrane further increase its effectiveness as a barrier. The multisubunit enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2), for example, consists of a transmembrane heterodimer (gp91^{phox}, p22^{phox}) and cytosolic subunits that assemble into the mature holoenzyme at this site; here, it generates microbicidal reactive oxygen species (ROS) to inhibit incoming pathogens (5).

Some intracellular pathogens, including enveloped and nonenveloped viruses, bacteria, protozoa, and fungi, do not cross the plasma membrane at the cell surface but are instead taken up into

vesicles. Although this strategy facilitates entry into cells, it does not provide direct access to their cytosol. Thus, pathogens entering the endocytic network must quickly subvert their vacuoles to avoid transport to the degradative lysosomal compartment or must escape into the cytosol. Bacteria, in particular, have evolved sophisticated systems to manipulate vesicle maturation by secreting effector proteins into the host cytosol in an attempt to establish a safe replicative niche for the pathogen (9). Host cells counteract this strategy by using compartmental PPRs to detect vesicular pathogens and to stimulate LC3-assisted phagocytosis (LAP) (6, 18). Viral exit from endosomes can also be blocked by the interferon-inducible transmembrane proteins (IFITM), which are effective against varied enveloped viruses including influenza, coronavirus, lentivirus, and flavivirus (5, 11).

Other compartmental borders include the nuclear envelope, which many viruses must also negotiate during their replication cycle, including adenovirus, HSV, and HIV. Except during cell division, the host genome is protected by the nuclear envelope, and traffic into and out of the nucleus is strictly controlled by the nuclear pore complex. Some viruses cannot pass through nuclear pores, while others rely on clever strategies for entry. For example, gammaretroviruses, like murine leukemia virus, cannot infect nondividing cells, but related lentiviruses, like HIV, can. Their ability to cross the nuclear envelope relies on hijacking nuclear import cofactors, including CPSF6, TNPO3, and nuclear pore proteins like NUP358 and NUP153 (19). Whether antiviral factors actively

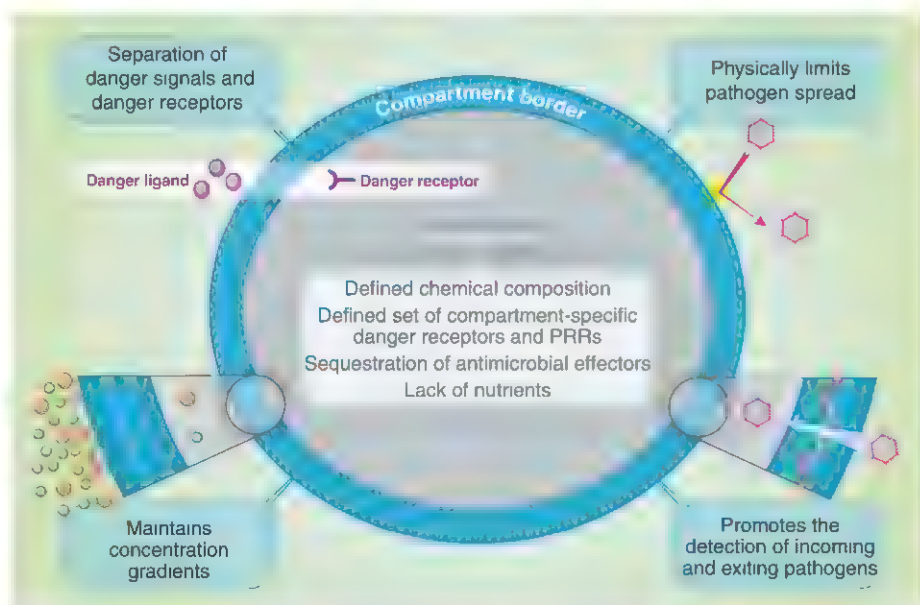


Fig. 1. Compartmentalization promotes cellular self-defense. Eukaryotic cells are composed of compartments separated by selectively permeable borders that control their composition. Cell and compartment borders can physically prevent pathogen invasion, and they house sensors that are “tripped” as pathogens try to cross them. Pathogen-induced damage to borders alters compartment composition; the resulting mislocalization of host molecules can be perceived as a danger signal. Control over compartment composition allows potent antimicrobial effectors that otherwise might damage the cell to be safely sequestered. Finally, each cellular compartment represents its own microenvironment that can be made hostile to pathogens.

prevent viral nuclear entry remains unclear, although the fact that the nuclear pore is significantly smaller (~10 to 20 nm) than most viral nucleocapsids (~20 to 100 nm) provides an effective antiviral barrier in its own right.

Compartmental borders represent a barrier both for incoming and exiting pathogens. Many enveloped viruses “bud” from the cell membrane, and several antiviral proteins inhibit this process, including tetherin and viperin (5, 11). Tetherin, a dimer comprising two long α -helices anchored at either end in the membrane, physically “tethers” the viral envelope to the plasma membrane as the virion buds, whereas viperin disrupts lipid raft microdomains used by viruses, such as influenza, to exit cells (5, 20).

How Compartmentalization Fosters Pathogen Detection by Danger Receptors

Cellular borders are not sufficient to prevent infection, and therefore, cells must have other ways of sensing and inhibiting pathogens. Cell-autonomous immunity relies on both PRRs to detect microbial signatures and danger receptors

that monitor DAMPs, i.e., disturbances in cellular homeostasis caused by infection, rather than the pathogen itself. The latter process predominates in organisms that lack circulating immune cells, such as plants and nematodes, and give rise to mechanistic models known as the “guard” hypothesis and effector-triggered immunity (14, 21).

Compartmentalization is a prerequisite for DAMP recognition, because only strict internal organization allows the detection of molecules outside their proper spatial context. Galectins are a family of cytosolic lectins with specificity for β -galactosides that detect damage to endosomes or lysosomes when luminal glycans, otherwise hidden inside vesicles, become accessible to the cytosol (Fig. 2). Galectins perceive even sterile damage to membranes and, thus, serve as versatile proxies of membrane damage caused by a broad spectrum of intracellular pathogens, including Gram-negative *Salmonella*, *Shigella*, and *Legionella*; Gram-positive *Listeria*; and non-enveloped viruses, such as adenovirus (22–25). Although the precise function of most galectins

remains to be established, recruitment of galectin-8 to damaged *Salmonella* Typhimurium-containing vacuoles brings in the autophagy cargo receptor NDP52, which induces antibacterial autophagy by means of LC3C to restrict bacterial proliferation (23, 26, 27).

Membrane damage also serves as a danger signal for different forms of programmed cell death (PCD) to limit pathogen spread. Plant cells have long been known to enlist this method against phytopathogens (21), but animals could potentially use it as well. Stimulation of sensory inflammasome complexes by membrane-disrupting bacteria induces pyroptosis, a form of PCD reliant on caspase-1 and/or -11 signaling, to inhibit bacterial growth in a cell-autonomous fashion (22, 28).

DAMP-mediated defense is not confined to detecting the altered redistribution of intracellular self-components. Translocation of extracellular antibodies into the host cell during infection can also trigger a protective response. Serum antibodies bound to bacteria or nonenveloped viruses are carried into the cytosol where they are detected by TRIM21, a cytosolic mammalian Fc receptor and E3 ubiquitin ligase (Fig. 2) (29). This detection triggers the synthesis of lysine 48 (K48) and K63 ubiquitin chains, which target virions for degradation

by the proteasome and AAA adenosine triphosphatase (ATPase) valosin-containing protein (VCP), and activates nuclear factor κ B (NF κ B), activating protein-1 (AP-1), and interferon regulatory factors IRF3, 5, and 7, respectively, which induce a potent antiviral state (30, 31). TRIM21 exhibits broad-spectrum defense as a result of antibody diversity and links cell-intrinsic defense with adaptive immunity.

The evolutionary advantage of DAMP-mediated cellular defense lies in its independence of pathogen structures. PAMPs may become invisible to a given PRR after genetic or chemical modification; however, avoiding detection by danger receptors is difficult for pathogens that rely on pore- or toxin-mediated membrane damage to enter the cell or escape from the phagosome. In addition, because DAMPs are not pathogen-specific, they offer protection against “new” pathogens, like emerging zoonotic viruses for which a given host may lack PRRs (14).

How Compartmentalization Fosters Pathogen Detection by PRRs

In bacteria, foreign DNA is sensed and destroyed by the CRISPR system and restriction endonucleases

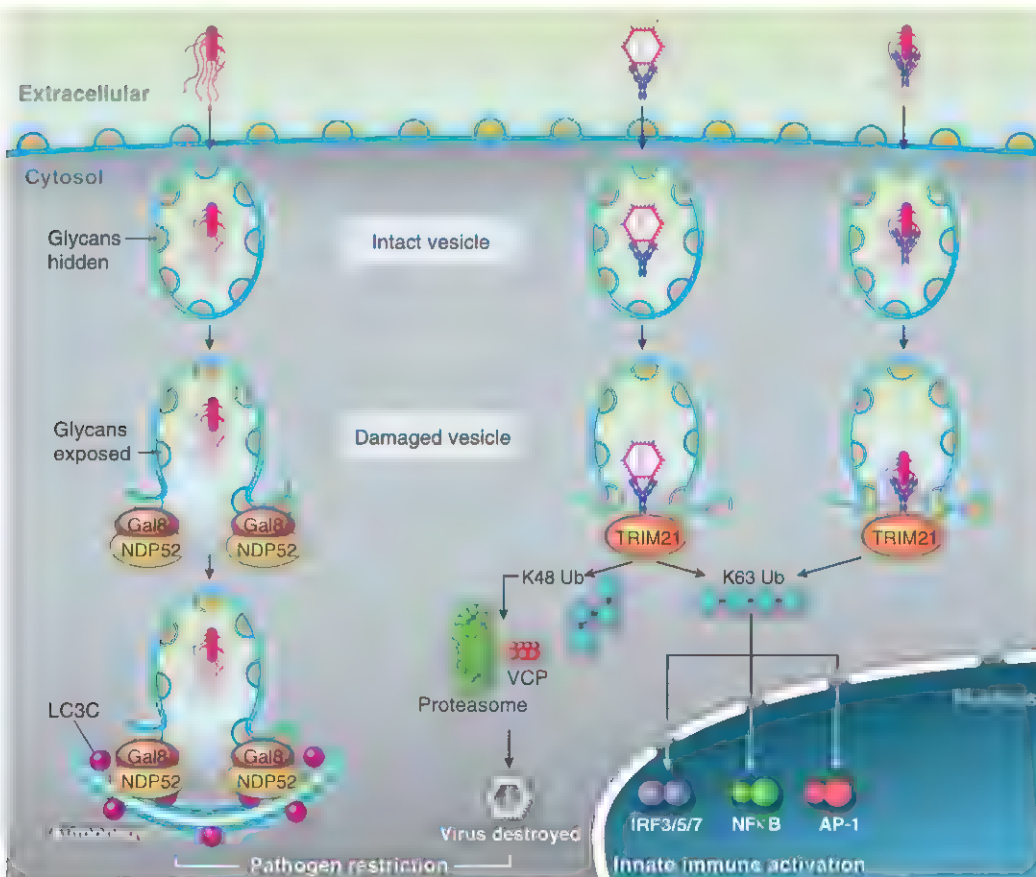


Fig. 2. Breakdown of compartment borders generates DAMPs, which trigger potent antimicrobial defenses. Membrane damage causes the translocation of extracellular molecules into the cytosol, which cells sense and interpret as a danger signal. Host glycans on burst phagosomes are detected by the cytosolic lectin galectin-8, whose accumulation provides an eat-me signal for the autophagy cargo receptor NDP52, causing LC3C-dependent autophagy and restriction of bacterial proliferation. Antibodies bound to bacteria and nonenveloped viruses are translocated into the cytosol upon release of pathogens from their internalized compartment. Cytosolic antibodies are detected by the E3 ligase and Fc receptor TRIM21, which targets virions for degradation by the proteasome and activates innate immune signaling.

(2). Because recognition motifs for most restriction endonucleases occur frequently in the host's own genome, these enzymes are paired with matching methyltransferases, which modify host DNA to demarcate it as "self." In eukaryotic cells, rather than being modified, DNA is largely sequestered inside the nucleus, which fosters the detection of foreign DNA in other compartments and allows the deployment of enzymes that mutate and/or degrade DNA without risk to the host genome. The abundance of cytosolic DNA receptors and sensors—such as AIM2, DAI, DNA-PK, IFI16, LRRFI1, and cyclic guanosine monophosphate-adenosine monophosphate (cyclic GMP-AMP or cGAMP) synthase—underscores the idea that packaging DNA into the nucleus provides an important advantage to host cells against invading pathogens by physically separating self from nonself (32–34).

Cytosolic RNA polymerase III also contributes to DNA sensing by transcribing AT-rich double-stranded DNA into uncapped RNA that is subsequently recognized by RIG-I (11). The addition of caps to the 5' end of mRNAs and other RNA modifications help distinguish host from foreign nucleic acids. Here, the lack of 5' N-7- or 2'-O-methyl-guanosine on unprocessed viral RNAs betrays their presence in the cytosol, just as a lack of 3' polyadenyl groups may reveal the existence of bacterial mRNA species (35, 36). Thus, host cells have devised a number of strategies to detect microbial DNA and RNA in the cytosol.

DNA or RNA occurring in the endolysosomal system is also conspicuously out of place. In mammals, compartmentalized Toll-like receptors (TLRs) detect pathogens that reside within this network (10). TLRs 3, 7, 8, and 9 are delivered by endoplasmic reticulum (ER) chaperones plus sorting adaptors to endosomes and lysosomes for sensing foreign nucleic acids (10). These luminal TLRs remain inactive until their ectodomain is cleaved by proteases in the endolysosomal system, a possible safeguard against activation by self RNA and DNA (10). Their strategic location also enables sampling of genetic material exposed after endolysosomal degradation. Targeting PRRs to compartments that harbor their respective nucleic acid ligands thus enables immune surveillance of its enclosed cargo.

Besides DNA and RNA, structural PAMPs also elicit strong cellular self-defenses. Translocation of

flagellin or rod components of bacterial type III secretion systems into the cytosol elicits robust NAIP-NLRC4 inflammasome activity, interleukin-1 β production, and pyroptosis (37, 38). Viral capsids are detected by a different set of cell-intrinsic proteins. TRIM5 α and TRIMCyp target the capsids of primate lentiviruses, including HIV (11). TRIMCyp binds directly to the capsid by means of a C-terminal CypA-like domain, which it obtained by gene duplication from CypA, an HIV host cofactor. The need for HIV to preserve binding to CypA poses a substantial hurdle for the virus to avoid detection by TRIMCyp, a problem compounded by the ability of certain TRIMCyps to isomerize between multiple conformations that are complementary to different lentiviruses

(39). TRIM5 α targets viral capsids by means of a C-terminal PRYSPRY domain containing six flexible loops reminiscent of antibody CDRs. Once bound, TRIM5 α uses the repetitive nature of the capsid structure as a template to form higher-order multimers and eventually a complete lattice, which prematurely uncoats the virus (11). Because capsid binding by TRIM5 α also activates innate immune signaling, it represents both a sensor and terminator of viral invasion (11).

How Compartmental Detection Promotes Pathogen Elimination

Host cells have evolved methods to link pathogen detection with pathogen disposal. One of the best examples is the recognition of cytosolic

pathogens and their subsequent delivery to lysosomes by the process of macroautophagy (6, 18). Macroautophagy sequesters cytosol destined for degradation into a de novo generated, membrane-bound compartment, the autophagosome. Autophagosome biogenesis proceeds by means of crescent-shaped membrane structures (phagophores) that grow around and, ultimately, enclose portions of the cytosol (18). During infection, eukaryotic cells co-opt autophagy to defend the cytosol against bacterial invaders, to attack microbe-containing vacuoles, and to remove pathogen-derived inclusion bodies (5, 6, 18). Selective autophagy relies on cargo receptors that cross-link "eat-me" signals on prospective cargo to ubiquitin-like proteins of the ATG8 family displayed on the phagophore membrane. Antimicrobial autophagy ("xenophagy") uses many of the same eat-me signals that alert the autophagic machinery to engulf host protein aggregates and damaged organelles (6, 18).

Autophagy efficiently restricts the subpopulation of vesicle-inhabiting bacteria that, by damaging the limiting membrane of their vacuole, become exposed to the cytosol, for example, *Mycobacterium tuberculosis* and *Salmonella Typhimurium* (18). Professional cytosol-dwelling pathogens like *Listeria monocytogenes* avoid such attacks [see review in this issue (40)]. Phagophore recruitment to bacteria depends on three nonredundant cargo receptors, i.e., NDP52 (41), p62 (42), and optineurin (43). Their bacteria-associated eat-me signals arise from (i) active ubiquitin deposition by host cells around susceptible bacteria, for example, by the E3 ligase LRSAM1 (6, 18, 44); (ii) cytosolic exposure of intravesicular glycans upon bacterial damage of the

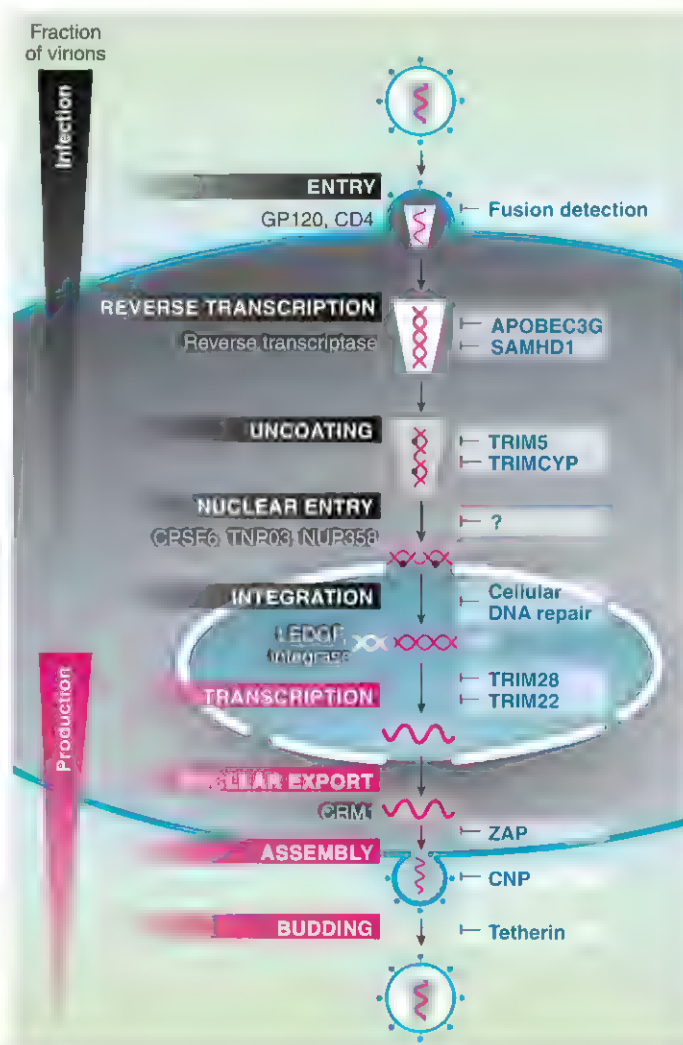


Fig. 3. Multilayered defenses synergistically inhibit infection and pathogen replication. To infect cells and complete their life cycle, retroviruses, such as HIV-1, must pass through multiple compartment borders and access distinct cellular compartments. Virions must recruit and retask cellular cofactors to negotiate their way through the cell and to replicate successfully. Meanwhile, antiviral factors adapted to specific cellular microenvironments target and inhibit specific steps of the viral life cycle. At each stage, only a fraction of virions are successful, which provides a highly synergistic defense system capable of inhibiting even quickly evolving pathogens. LEDGF, PC4 and SFRS1 interacting protein-1; ZAP, zinc finger CCCH-type antiviral protein-1.

vacuolar membrane, bound by galectin-8 and recruiting NDP52 (23); or (iii) release of mycobacterial DNA after phagosome permeabilization by the type VII secretion system ESX-1, detected by STING and causing ubiquitin-deposition (45). In the case of viruses, capsids may be directly bound by p62 to activate the autophagic cascade (46), which facilitates the removal of toxic capsomere aggregates from infected cells and tolerizes them against the pathogenic consequences of infection, consistent with the idea of disease tolerance as a defense strategy (47).

Besides sequestering cytosolic pathogens, cargo receptors also select cytosolic proteins for autophagy that yield antimicrobial peptides upon digestion, which contribute to the bactericidal properties of autophagosomes (48). Interferon (IFN)-inducible 65-kD guanylate-binding proteins (GBPs) help traffic p62-bound cytosolic proteins to autophagic vacuoles for generating bacteriolytic peptides (49). GBPs also directly target bacteria and protozoa in damaged vacuoles or after escape into the cytosol for subsequent elimination; how they recognize these pathogens is unknown (4, 5, 49, 50). Members of the related IRG family of immune GTPases target vacuolar pathogens by recognizing self-components on these structures. Irgm1 binds phosphatidylinositol 3,4,5-trisphosphates generated by host class I phosphatidylinositol 3-kinases on mycobacterial phagosomes to assemble soluble NSF attachment protein receptor (SNARE) and autophagy-related proteins for fusion with lysosomes (4, 5, 51). Other IRGs require Atg5 and GBPs to target *Toxoplasma* vacuoles, which then undergo disruption (50, 52). Thus, it appears both self and nonself signals solicit autophagy receptors and IFN-inducible GTPases to eliminate intracellular pathogens as part of the cell-autonomous defense program.

How Cells Control Compartmental Composition to Limit Microbial Growth

Controlling compartment composition allows cells to create conditions unfavorable for microbial growth. For example, the concentration of deoxynucleotide triphosphates (dNTPs) in the cytosol is significantly higher in virally permissive than in certain nonpermissive cell types (~40 to 70 nM versus ~1 to 15 μ M) (53). In order to remove excess dNTPs, human dendritic cells and macrophages express an IFN-inducible deoxyguanosine triphosphate triphosphohydrolase, SAMHD1, which hydrolyzes dNTPs to block reverse transcription and cDNA synthesis in HIV-1 (53). Another compositional strategy to restrict microbial growth is depletion of amino acids, exemplified by the IFN-induced indoleamine 2,3-deoxygenase (IDO) which degrades L-tryptophan. IDO potently inhibits human viruses (HSV and hepatitis B virus), bacteria (*Chlamydia*, *Francisella*, and *Rickettsia*) and protozoan parasites (*Leishmania*, *Trypanosoma*, and *T. gondii*) (5).

Perhaps nowhere else in the cell is control of compartmental composition more effective than

in phagolysosomes and autophagolysosomes, acidified organelles designed to kill and degrade internalized pathogens. Their sterilizing power comes from the concerted action of several factors. Mammalian cells express proton-dependent efflux pumps, such as natural resistance-associated macrophage protein-1 (NRAMP1), that export Mn^{2+} and Fe^{2+} from vacuoles to prevent access of captured microbes to these essential metals (5). Antimicrobial peptides disrupt the outer envelope of pathogens, and luminal proteases, lipases, and glycosidases imported through the Golgi-late endosome pathway further degrade this material. Reactive oxygen and nitrogen species oxidize and nitrosylate, respectively, pathogen lipids, DNA, and proteins (5). Together, they create a hostile environment for most incoming pathogens. The low pH (~4.5 to 5.0) generated inside these compartments by a proton-pumping vacuolar ATPase and maintained by antiporters, such as the sodium-hydrogen exchanger-1 (NHE1), is optimal for lysosomal hydrolase activity and the conversion of superoxide (O_2^-) to H_2O_2 and of nitrogenous end products back to the toxic radical, nitric oxide (NO). Import of copper ions by the P-type ATPase Cu^{2+} pump ATP7A promotes the formation of toxic hydroxyl ($\cdot OH$) radicals (5). Concentrating diverse antimicrobial activities in a specialized organelle thus promotes microbial killing, with the pH difference between lysosomes and cytosol safeguarding the cell against the consequences of potential lysosomal rupture.

How Multilayered Self-Defense Effectively Restricts Invading Pathogens

The multiple barriers and compartments that define cellular architecture provide a series of obstacles, all of which pathogens must overcome in order to replicate. Therefore, although the protection provided at each stage is not complete, their combination is extremely effective, as illustrated for HIV in Fig. 3. Although a cell might be challenged by many infectious particles, the fraction that navigates each step is small, cumulatively reducing the probability of a productive infection. Some virions do not correctly engage with their receptor and co-receptor and thus fail to deposit their capsid into the cytosol. Virions that begin reverse transcription are substrates for APOBEC3G, a host restriction factor that mutates the viral genome by deaminating deoxycytidine (11). Reverse transcription is also targeted by SAMHD1, which depletes dNTPs (53). Meanwhile, as the capsid traverses the cytosol, TRIM5 α and TRIMCyp interfere with its uncoating (11). Virions that make it past these defenses have to negotiate nuclear entry by using cofactors to pass through the pore. Once inside the nucleus, the viral genome becomes a substrate for host DNA repair enzymes, which circularize it into long terminal repeat 1-LTR or 2-LTR circles (54). Viral genomes can also be degraded by DNA repair enzymes XPB and XPD (55). Even integration does not yet constitute successful completion of the retroviral "life cycle." Transcription

of integrated virus is repressed by TRIM22 and TRIM28 (56, 57), while particle assembly and viral budding are inhibited by 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) and tetherin, respectively (20, 58).

In conclusion, the organization of eukaryotic cells into compartments has driven the evolution of highly effective cellular self-defense, which has forced pathogens to adapt to each environment they encounter. Not only must pathogens survive these environments, but they must also have means of transportation between them. Controlling the movement of pathogens is therefore another defense strategy, realized, for example, by the enclosure of professional cytosol-dwelling bacteria into septin cages that prevent bacteria from usurping the host actin cytoskeleton for mobility and cell-to-cell spread (59).

Outlook

This Review has focused on how cells use their spatial organization to defend themselves against infection, although the temporal sequence of events is equally important. A cell invaded in the first few hours of pathogen exposure relies on preformed defense factors (11). However, after these first few hours, innate immune signaling activates additional cell-autonomous responses, as exemplified by IFN-induced protection that elicits transcription of hundreds of new genes (5). Remarkably, we know almost nothing about the protein functions encoded by the vast majority of these genes. After this innate response, tailor-made adaptive immunity helps clear pathogens and prevent persistence. Again, we now realize that innate and adaptive immunity may not be so easily demarcated. This point is well illustrated by the Fc receptor TRIM21, which recognizes antibody-opsonized pathogens in the host cell cytosol (31). It is also becoming clear that traditional immunity must cooperate with cells outside the immune system for optimal protection of the host. Such cells are often endowed with potent antimicrobial capacity. Neurons, for example, exhibit powerful antiviral programs against a wide spectrum of RNA viruses (46, 60). Our attempts to understand the functional anatomy of this "nonclassical" immune system will thus represent a major scientific frontier in the years ahead.

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Morals and Markets

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The possibility that market interaction may erode moral values is a long-standing, but controversial, hypothesis in the social sciences, ethics, and philosophy. To date, empirical evidence on decay of moral values through market interaction has been scarce. We present controlled experimental evidence on how market interaction changes how human subjects value harm and damage done to third parties. In the experiment, subjects decide between either saving the life of a mouse or receiving money. We compare individual decisions to those made in a bilateral and a multilateral market. In both markets, the willingness to kill the mouse is substantially higher than in individual decisions. Furthermore, in the multilateral market, prices for life deteriorate tremendously. In contrast, for morally neutral consumption choices, differences between institutions are small.

It is a pervasive feature of market interaction to impose costs on uninvolved third parties. Producing and trading goods often creates negative externalities, such as detrimental working conditions for workers, possibly associated with reduced life expectancy, child labor, suffering of animals, or environmental damage. People who participate in markets by buying such goods often seem to act against their own moral standards. The risk of moral decay through market interaction has been discussed in politics, ethics, and in the social sciences (1–7). Observing that with technological progress and the increasing ubiquity of market ideas, markets continue to enter further and further into domains of our social life (8), political philosopher Michael Sandel has recently reemphasized this critique, stating that “we have to ask where markets belong—and where they don’t. And we can’t answer this question without deliberating about the meaning and purpose of goods, and the values that should govern them” (9). The relationship between markets and values has received attention both in theoretical work (10, 11) and in empirical cross-sectional studies that compare the level of prosociality across different market societies and cultures (12–14). Identifying a causal effect of markets on values is difficult with cross-sectional or historical data, however, simply because institutions and values coevolve. Moreover, comparing values across societies implies comparing a set of multiple institutions at the same time with unknown and possibly interacting features. For example, markets are observed in very different legal systems, which renders the isolation of the effects of “markets” across societies extremely difficult. For these reasons, we implemented a controlled environment by randomly assigning subjects to different institutions. This allows identifying a causal effect of institutions on outcomes.

Our evidence shows that market interaction causally affects the willingness to accept severe, negative consequences for a third party.

The Mouse Paradigm

Our paradigm for studying moral values and detrimental effects on third parties is the trade-off between a mouse life and money. In our main treatments, human subjects faced the decision to either receive no money and to save the life of a mouse, or to earn money and to accept the killing of a mouse. This paradigm involves a drastic and irreversible decision and is well suited for studying moral conflict: Although the content of morality is culturally determined and time and space contingent, there exists a basic consensus that harming others in an unjustified and intentional way is considered as immoral (15).

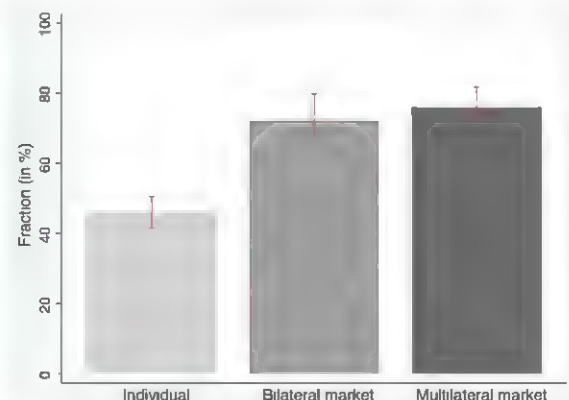
In all treatments of the experiment (16), which was approved by the Ethics Committee of the University of Bonn, subjects were explicitly informed about the consequences of their decision. They knew that their mouse was a young and healthy mouse, which in case it survived would in expectation live for about 2 years in an appropriate, enriched environment, jointly with

a few other mice. For illustrative purposes, we presented to subjects the picture of a mouse on an instruction screen (fig. S1). The instructions informed subjects explicitly about the killing process, in case they decided to kill their mouse. The killing process was also shown in a short video that was presented to subjects (17).

The mice used in the experiment were so-called “surplus” mice: They were bred for animal experiments, but turned out to be unsuited for study, e.g., because some specific gene manipulation had failed. They were perfectly healthy, but keeping them alive would have been costly. Although it was true that the mice would live or be killed based on the decisions of subjects in the experiment, the default for this population of mice was to be killed, as is common practice in laboratories conducting animal experiments. Subjects were informed explicitly about the default in a postexperimental debriefing (18). Mice that were chosen to survive because of subjects’ decisions were purchased by the experimenters and kept in an appropriate, enriched environment. Thus, these mice survived precisely as stated in the instructions. As a consequence of our experiment, many mice that would otherwise have been killed right away were allowed to live for roughly 2 years.

Markets are institutions where sellers and buyers interact and can trade items. Trade occurs whenever a seller and a buyer agree on a price. For our main result, we analyzed three different conditions (see table S1): an individual treatment in which subjects decided between the life of their mouse and a given monetary amount, a bilateral trading market, and a multilateral trading market. Treatment assignment was random. The individual treatment serves as a benchmark and comparison standard for decisions made in markets. The bilateral market is the most basic form of a market situation with one buyer and one seller bargaining over prices in order to trade. In the multilateral market, many buyers and sellers potentially trade with each other. In comparing

Fig. 1. Market interaction erodes moral values, relative to individually stated preferences: fractions of subjects who are willing to kill a mouse for monetary amounts below or equal to 10 euros in the individual treatment, the bilateral market, and the multilateral market. For both markets, fractions are calculated using the lowest prices accepted by sellers in actually concluded trades. Error bars show standard deviations at the means. Differences between the individual treatment and markets are significant at the 1% level. Individual versus bilateral market: $P < 0.01$, $n = 160$ (two-sample test of proportions). Individual versus multilateral market: $P < 0.01$, $n = 178$ (two-sample test of proportions). The difference between markets is not statistically significant.



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decisions from the individual treatment to decisions made in markets, we abstract away from the question of whether a good is priced at all. In all treatments, subjects could exchange life for money.

In the individual treatment, subjects faced a simple binary choice, labeled option A and option B. Option A implied that the mouse would survive and that the subject would receive no money. Option B implied the killing of the mouse and receiving 10 euros. This treatment informs us about the fraction of subjects who are willing to kill the mouse for 10 euros. One hundred and twenty-four subjects participated in this treatment.

To study markets, we implemented the so-called double auction market institution, which is widely used in economics to investigate market outcomes [for an overview, see (19)]. In the bilateral double auction market, one seller and one buyer bargained over killing a mouse for a total gain of 20 euros that the two parties could split up between themselves. The seller was endowed with a mouse. As in the individual treatment, he or she was explicitly told that the “life of the mouse is entrusted to your care.” Bargaining over the 20 euros was conducted during a continuous auction, i.e., buyer and seller could make as many price offers as they liked (16). If a buyer and a seller agreed on a trade, the buyer received 20 euros minus the price agreed upon. The seller received the price. In addition, the mouse of the seller was killed, reflecting a situation in which trade takes place to the detriment of a third party. If a seller or a buyer did not trade, earnings for both were zero and the mouse survived. A seller in the bilateral market was in the same situation as a subject in the individual treatment in that he or she could either refuse a monetary amount or accept a monetary amount and kill a mouse. Subjects were told that no market participant was forced to make price offers or to accept an offer, that their mouse would be killed only if a trade occurred, and that the mouse would survive if they decided not to trade. There were 10 trading periods. Seventy-two subjects participated in this treatment.

The multilateral double auction market treatment was exactly like the bilateral market treatment, except that in this condition seven buyers and nine sellers bargained over prices (16). The nine sellers were all endowed with one mouse each. Subjects on both sides of the market could make as many price offers as they liked. All subjects could accept a price offer from the other side of the market. Available price offers of both market sides were always shown on a screen. Once a price offer of a trader was accepted, trade occurred implying the killing of a mouse. Payoff consequences were identical to those of the bilateral market. There were 10 periods. We ran six sessions with a total of 96 subjects.

To allow for further analyses, we ran several additional treatments (for details see below). In the individual price-list treatment, we offered subjects a menu of prices to elicit the monetary amount needed to pay subjects to make them

indifferent between killing and receiving money. To establish a benchmark in terms of how markets affect morally neutral values, we conducted an individual price-list treatment and a multilateral market treatment analogously to the mouse treatments, but for a consumption good. Finally, we ran two further control treatments based on the individual treatment. In sum, we ran nine treatments with a total of 787 subjects.

Our key hypothesis was that markets would display a tendency to erode moral standards, relative to individual decision-making, because of three essential features of market interaction. First, in markets, it takes two people who agree on trading to complete a trade, implying that responsibility and feelings of guilt may be shared and thus diminished (20, 21). Second, market interaction reveals social information about prevailing norms. Observing others trading and ignoring moral standards may make the pursuit of self-interest ethically permissible, leading further individuals to engage in trade. In addition, the mere existence of a market may provide social information about the appropriateness of trading, rendering the killing of mice more allowable (22, 23). Third, markets provide a strong framing and focus on materialistic aspects such as bargaining, negotiation, and competition, and may divert attention from possible adverse consequences and moral implications of trading (11, 24). In contrast to our market conditions, subjects in the individual condition do not interact with other subjects and therefore receive no social information, do not share responsibility if they trade, and are not exposed to a market framing.

These three features are present in all markets, even in simple bilateral markets. In addition, in the multilateral market with its presence of competing sellers, the notion of being pivotal may be diffused as well (25); unless a seller cares specifically about his own mouse, he may argue that if he does not trade his mouse with some buyer, another seller may conclude the trade with that buyer, selling and killing his mouse. This common feature of markets may make subjects feel less responsible, rendering it more difficult to sustain moral values even if values per se remain

unchanged. In sum, we therefore expected a higher willingness to kill in the bilateral and the multilateral market compared to individual decision-making. In addition, owing to notions of being less pivotal, the killing rate was expected to be even higher in the multilateral than in the bilateral market. We further hypothesized that the decay of moral values would also be reflected in prices, such that mice would be killed for lower prices in the market treatments compared to the individual treatments. Finally, we studied markets where the cost of trading involves opportunity costs of consumption rather than moral costs. For these morally neutral consumption good markets, we hypothesized no decline of values through market interaction.

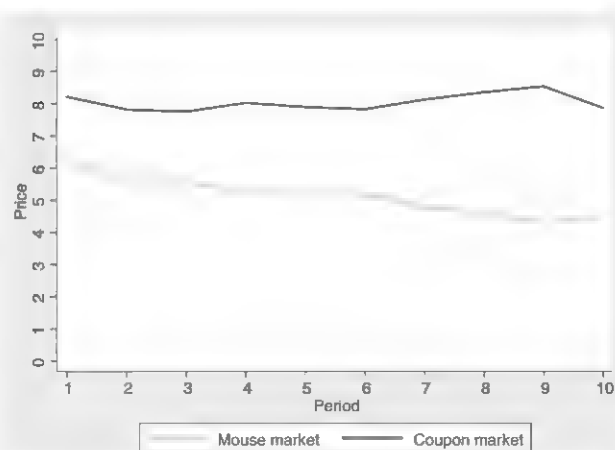
Markets Erode Moral Values

Figure 1 shows our main result. Given our interest in studying the effects of institutions on moral valuations in a given population, we compare the fractions of subjects who are willing to agree to the killing in the individual treatment, the bilateral market, and the multilateral market for monetary amounts below or equal to 10 euros (26). For both markets, fractions are calculated with the lowest prices accepted by sellers in actually concluded trades. We focus on lowest accepted prices to approximate from above sellers' reservation values for killing a mouse.

In the individual decision treatment, 45.9% of subjects were willing to kill their mouse for 10 euros. In contrast, 72.2% of sellers in the bilateral market were willing to trade for prices below or equal to 10 euros. The increase in willingness to kill relative to the individual condition is statistically significant ($P < 0.01$, $n = 160$, two-sample test of proportions) (16). In the multilateral market, the willingness to kill was also substantially higher compared to the individual condition: 75.9% of sellers were willing to kill a mouse for less than or equal to 10 euros ($P < 0.01$, $n = 178$, two-sample test of proportions). This is actually a lower bound because in a given period, only seven of the nine sellers could trade at all.

To provide a more detailed understanding of the effects of markets on morals, we implemented

Fig. 2. Evolution of trading prices in the multilateral mouse market and the multilateral coupon market (means over all trades). The downward trend in prices in the mouse market is significant ($P = 0.006$, $n = 297$, random effects regression). No significant price trend is observed in the coupon market ($P = 0.319$, $n = 233$, random effects regression).



an additional individual treatment, the individual price-list treatment. This treatment informs us about how much money subjects would need to receive in the individual condition to yield a similarly high killing rate as in markets. In this treatment, subjects faced an increasing price-list, which is a standard procedure for eliciting individual values and preferences in an incentive-compatible way. As in the individual treatment, subjects were shown a list of binary alternatives, labeled option A and option B. Option A implied that the mouse would survive and that the subject would receive no money. Option A was the same in each decision row. Option B implied the killing of the mouse and the receipt of a monetary amount. Monetary amounts associated with killing the mouse increased from row to row, starting from 2.50 up to 50 euros, in steps of 2.50. Subjects were informed that one choice situation would be randomly selected after all choices had been made. The choice in this situation would be implemented, including payment consequences and, in case option B had been chosen, the killing of the mouse. The switching point from option A to option B informs us about the minimum monetary amount that makes a subject willing to kill the mouse, i.e., the moral value attached to the life of the mouse. The earlier a subject switches, the less he or she values the life of his or her mouse relative to earning money. Despite differences in elicitation procedures, including randomness of the selected choice, the fractions of subjects willing to kill for 10 euros or less were almost identical between the individual and the individual price-list treatment (45.9 versus 42.7% of subjects, respectively; $P = 0.636$, $n = 220$, two-sample test of proportions) (fig. S2). Ninety-six subjects participated in the individual price-list treatment.

As shown above, in the bilateral trading market, 72.2% of sellers were willing to trade for prices below or equal to 10 euros. In comparison, in the individual price-list treatment, a similarly high willingness to kill (71.9%) was reached only for monetary amounts of 47.50 euros. Thus, it is necessary for subjects to receive considerably more money in the individual than in the market condition to observe a comparable willingness to kill. Turning to the multilateral market, a similar picture emerges. Here the killing rate was 75.9% for prices below or equal to 10 euros. A similar rate in the individual price-list treatment would require paying subjects monetary amounts above 50 euros. In line with our hypothesis, actual prices in the multilateral market were much lower than 10 euros, however (Fig. 2). The overall average price level was only 5.1 euros (27). In the individual price-list condition, the fraction of subjects who were willing to kill the mouse for 5 euros was only 34.4%. Thus, for prices that actually evolved in the multilateral market, the willingness to kill was much higher than in the individual price-list condition.

The price-list treatment can also be used to illustrate the decay in valuations in terms of the

predicted fraction of trade (16). Assuming that valuations in the price-list condition and the bilateral market were the same, we can use valuations from the price list to simulate the predicted trade probability in the bilateral market. The simulated trade fraction is 25.9%, which is in sharp contrast to the actually observed trade frequency of 47.7% in the bilateral market ($P < 0.01$, $n = 168$, two-sample test of proportions). This provides a further confirmation that valuations for mice have declined considerably.

Moral Versus Morally Neutral Values

The final step of the analysis compares decay in moral versus morally neutral values. We hypothesized that for moral values the decay is more pronounced than for private consumption values, where trading involves opportunity costs of consumption rather than costs to third parties. To test this, we ran two additional treatments, identical to the multilateral market and the individual price-list treatment but using consumption goods. The good we considered was a coupon that could be used to buy products at the merchandising shop of the University of Bonn (16). In both treatments, the price-list and the market treatment, subjects were endowed with a coupon. In case they accepted a monetary amount (in the price-list condition) or decided to trade (in the market condition), they had to return their coupon, which was then invalidated. Parameters, instructions, and procedural details were identical to the mouse treatments. Thus, consequences were similar in the mouse and the coupon treatments, except that in the latter, the cost of trading involved opportunity costs of consumption rather than moral costs, i.e., loss and invalidation of a coupon versus killing of a mouse.

To assess the effect of markets on moral versus private consumption values, we use valuations from the individual price-list conditions and compare them to valuations in the respective multilateral markets (16). The dependent variable is a subject's minimum trading price. Running Tobit and interval regressions, we find that in the mouse treatments, there is a strong negative and statistically significant effect of market interaction. Thus, for a given monetary amount, subjects reveal a higher willingness to kill in markets than in the individual condition. For coupons, the effect of markets is much smaller and insignificant. We also find that the effects of markets differ significantly between mice and coupons (16). In addition, we observe a difference in the price dynamic between multilateral mouse and coupon markets (Fig. 2). In the mouse market, average prices start at rather low levels (compared to the individual condition) and decline from 6.4 euros in the first period to levels as low as 4.5 euros in the final period. This decline in prices is statistically significant ($P = 0.006$, $n = 297$, random fixed effects regression). The downward trend provides a further indication of moral decay in the mouse market and is suggestive of social learning and endogenous social norm formation.

Intuitively, observing low trading prices in the market may make it normatively acceptable to offer or accept low prices as well (16). In contrast to the downward trend in prices in the mouse market, no significant price trend is observed in the coupon market ($P = 0.319$, $n = 233$, random fixed effects regression). The analysis thus reveals a systematic difference between markets involving moral versus morally neutral values: When identical procedures, parameters, and market institutions are used, moral values decline significantly more than values that are morally neutral.

Whereas prices decline in the multilateral mouse market, trade volumes in both bilateral and multilateral markets are constant across periods, suggesting that a number of subjects were not tempted to engage in trading. Apparently, markets did not erode values of all subjects (16). We speculate that subjects who refused to exchange money for mouse life at all may have followed a rule-based, e.g., Kantian, ethic: "... everything has either *price* or *dignity*. Whatever has price can be replaced by something else which is *equivalent*; whatever, on the other hand, is above all price, and therefore admits of no equivalent, has a *dignity*" (16, 28).

Robustness and Discussion

Three potential concerns may be raised with respect to our main finding. First, one could argue that we observe the main treatment effect because total surplus was greater in markets than in the individual condition (20 versus 10 euros). If traders dispose of social preferences, they may have cared not only about their own payoff but also attached some value to the payoff of the other trader (buyer). We therefore ran a control condition, which was identical to the individual condition but in which we introduced a second passive participant. One hundred and sixteen subjects took part in this control treatment, with 58 subjects participating in the role of active decision-makers. A passive participant received 10 euros if the active participant decided to kill the mouse (such that the death of a mouse generated a total surplus of 20 euros as in the market treatments). The observed fraction of killing among subjects in the active role is 44.8%. This fraction is significantly different from fractions in both market conditions (bilateral market, $P = 0.009$, $n = 94$, and multilateral market, $P = 0.001$, $n = 112$, two-sample test of proportions). Furthermore, this fraction is remarkably similar to the individual condition ($P = 0.890$, $n = 182$, two-sample test of proportions).

Second, subjects may have perceived killing the mouse as a side-effect of the act of trading in the market treatments, whereas in the individual treatment subjects may have perceived killing the mouse as a direct means to earn money. If this were the case, subjects may have found it more difficult to opt for killing in the individual treatment. We therefore ran another control treatment identical to the individual treatment but in which

subjects could buy a lottery ticket for 2 euros. This renders it more likely that subjects perceive the mouse death as a side-effect of a buying decision. The lottery paid out either 10 or 15 euros, respectively, both with 50% probability. We chose an expected net value of $12.50 - 2 = 10.50$ euros to compensate for possible risk aversion of subjects. If subjects bought the lottery ticket, a mouse got killed “as another consequence” of the buying decision, i.e., as a side-effect. Forty-three subjects participated in this additional control condition. Again, outcomes are very similar to those in the individual condition: 46.5% of subjects decided to buy the ticket accepting the killing of a mouse. This fraction is significantly different from fractions in both market conditions (bilateral market, $P = 0.021$, $n = 79$ and multilateral market, $P = 0.003$, $n = 97$, two-sample test of proportions). Unsurprisingly, the killing rate is not significantly different from the individual condition ($P = 0.946$, $n = 167$, two-sample test of proportions).

Third, let us comment on why we used the minimum trading price as our main dependent variable to assess a seller’s willingness to kill a mouse in markets [see also (16)]. Very likely, traders tried to negotiate higher prices than their reservation values to realize positive gains from trade. This should be the case for any market situation with information rents in which reservation values are private, as in our case. For example, a seller in the bilateral market with a reservation value of 5 euros is unlikely to actually trade at 5 euros. Instead, he should try to negotiate higher prices. We therefore think that concluded prices provide an upper bound for the sellers’ reservation values. One may also argue that using the minimum concluded price could bias results if sellers made mistakes, erroneously agreeing to trade at prices lower than they would have actually liked to accept. We believe that it is unlikely that traders made such mistakes, because trading involved a deliberate decision to either accept or make offers. Yet, accounting for this possibility, we also calculated median values of concluded trading prices below or equal to 10 euros. The corresponding killing fractions are 67% for the bilateral market and 76% for the multilateral market, very similar to the ones reported in Fig. 1. These fractions are statistically significantly different from the individual condition ($P = 0.029$ for bilateral market and $P < 0.001$ for multilateral market, two-sample test of proportions).

We stress another aspect of our results: following the methodological standards in experimental economic, it was essential to incentivize subjects’ decisions in the individual condition, i.e., subjects needed to receive money according to their decisions. Otherwise, a comparison with market outcomes would have been misleading. For subjects, it would be “cheap” to claim that they are moral if being moral costs nothing. The comparison of the individual treatment with markets did therefore not involve paying money

versus not paying money. Yet, introducing a money prime may already lower moral standards, as several studies have pointed out. For example, it has been shown that material primes or labels reduce helpfulness or prosocial behavior and increase competitiveness (29–31) and that an economics background correlates with selfishness (32). Hence, the impact of markets on moral behavior may in general be even more pronounced than our study suggests.

We have shown that market interaction displays a tendency to lower moral values, relative to individually stated preferences. This phenomenon is pervasive. Many people express objections against child labor, other forms of exploitation of the workforce, detrimental conditions for animals in meat production, or environmental damage. At the same time, they seem to ignore their moral standards when acting as market participants, searching and buying the cheapest electronics, fashion, or food, and thereby consciously or subconsciously creating the undesired negative consequences to which they generally object. We have shown that this tendency is prevalent already in very simple bilateral trading where both market sides are fully pivotal in that if they refuse to trade, the mouse will stay alive. In markets with many buyers and sellers, diffusion of being pivotal for outcomes adds to moral decay. This “replacement” logic is a common feature of markets, and it is therefore not surprising that the rhetoric of traders often appeals to the phrase that “if I don’t buy or sell, someone else will.”

In the experiment, subjects were fully aware of the consequences of their decisions in that they could save the life of a mouse if they refused to accept a monetary amount. Our findings therefore suggest that appealing to morality has only a limited potential for alleviating negative market externalities. For example, anti-child-labor or environmental protection campaigns may not be that effective because markets for goods undermine the relevant social values. The results also suggest why societies do ban markets for certain “repugnant” activities (33). Historically, dispute about the marketability and the appropriateness of markets has led to some of the most fundamental upheavals within modern societies. For example, the abolishment of trading human beings was a major issue in the American Civil War. Martin Luther’s critique of the trade of indulgences, in which buyers and sellers exchanged money for the freedom from God’s punishment for sin, was a key element of the Protestant Reformation. Karl Marx’s idea that capital stock should not be tradable, that it must belong to the workers themselves, is a cornerstone of communist ideology. With the recent financial crisis, discussion has arisen about the appropriateness of markets for complex financial products like derivatives involving high risks. Stock traders have been criticized for riding bubbles and for cashing in short-term profits without thinking about possible negative long-term impacts on companies, as well as on society in general.

Markets have tremendous virtues in their capability to generate information about scarcity and to allocate resources efficiently. The point of this study is not to question market economies in general. Indeed, other organizational forms of allocation and price determination such as in totalitarian systems or command societies do not generically place higher value on moral outcomes (34). Furthermore, the development of a complex market structure may require and therefore correlate with the prevalence of moral and social values, such as trust and cooperativeness. Results confirming this intuition, in line with the *Doux-commerce Thesis* (35), are expressed, e.g., by Kenneth Arrow (36). However, focusing on the causal effects of institutions, we show that for a given population, markets erode moral values. We therefore agree with the statement quoted at the beginning that we as a society have to think about where markets are appropriate—and where they are not.

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17. Comparable demonstration videos showing the killing process are publicly available at laboratories conducting animal research. The video presented to subjects showed how mice are gassed.
18. This information is irrelevant for the consequences of subjects’ decisions. If they choose to save their mouse, the mouse survives. If they decide to kill it, they receive money and the mouse is killed. We could have introduced another framing, informing subjects about the existence of “surplus” mice. Although this would not have changed any consequences, it may have changed the perception of the situation at hand, e.g., in line with evidence on the so-called omission-commission bias; see, e.g., (37).
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Supplementary Materials

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Supplementary Text
Figs. S1 and S2
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References (40, 41)

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Rational HIV Immunogen Design to Target Specific Germline B Cell Receptors

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Vaccine development to induce broadly neutralizing antibodies (bNAbs) against HIV-1 is a global health priority. Potent VRC01-class bNAbs against the CD4 binding site of HIV gp120 have been isolated from HIV-1-infected individuals; however, such bNAbs have not been induced by vaccination. Wild-type gp120 proteins lack detectable affinity for predicted germline precursors of VRC01-class bNAbs, making them poor immunogens to prime a VRC01-class response. We employed computation-guided, in vitro screening to engineer a germline-targeting gp120 outer domain immunogen that binds to multiple VRC01-class bNAbs and germline precursors, and elucidated germline binding crystallographically. When multimerized on nanoparticles, this immunogen (eOD-GT6) activates germline and mature VRC01-class B cells. Thus, eOD-GT6 nanoparticles have promise as a vaccine prime. In principle, germline-targeting strategies could be applied to other epitopes and pathogens.

Protection against disease by nearly all licensed vaccines is associated with induction of antibodies (1). Viruses with high antigenic diversity, such as HIV, influenza virus, and hepatitis C virus, pose major challenges for vaccine development (2). Most exposed surfaces on the Envelope glycoproteins (Env) of these viruses are hypervariable or shielded by glycans (3), and traditional vaccine approaches tend to induce neutralizing antibodies against only a small subset of viral strains (4–6). However, discoveries of bNAbs against each of these viruses have identified conserved epitopes as leads for vaccine design (2), and structural analysis has provided atomic definition for many of these epitopes (7, 8). Structure-based approaches are, therefore, needed to reverse-engineer vaccines capable of inducing bNAbs against these conserved epitopes (9).

High-potency VRC01-class bNAbs against the HIV gp120 CD4 binding site (CD4bs) have been isolated from several individuals infected with different strains of HIV-1 (10–12). VRC01-class bNAbs all derive from the human VH1-2*02 variable heavy gene but differ substantially in amino acid sequence and complementarity-determining region H3 (CDRH3) length and use a few different variable light chain genes (figs. S1 and S2). Structural studies have revealed that VRC01-class bNAbs employ a common mode of gp120 binding in which the VH1-2 framework mimics CD4 and provides additional electrostatic and hydrophobic contacts (Fig. 1A) (12–15). A short CDR3 loop is also required for interaction with gp120 V5 and Loop D, and a CDR1 deletion in many VRC01-class bNAbs avoids clashes with a glycan linked to Asn²⁷⁶ (N276) on loop D.

Vaccine design to induce VRC01-class bNAbs is attractive because VH1-2 genes are estimated to be present in ~2% of the human Ab repertoire (16) and, even considering restrictions on light chain usage, suitable precursors should be present in the naïve B cell repertoire of most individuals. However, predicted germline (GL) precursors for VRC01-class bNAbs exhibit no detectable affinity for wild-type Env (11, 13) (Table 1 and table S1), a potential explanation for the rarity of VRC01-class bNAbs in HIV-1 infection (13). More important, wild-type Env constructs lacking GL affinity are poor vaccine candidates to prime VRC01-class responses, because they are unlikely to reliably stimulate GL precursors to initiate antibody maturation.

Immunogen Design Strategy

To address the problem described above, we modified the CD4bs on a minimal, engineered outer domain (eOD) (17) to produce a germline-targeting vaccine prime (Fig. 1) with two important binding properties: (i) moderate affinity for multiple predicted VH1-2*02 GL-Abs to enhance the ability to activate VH1-2 GL B cells with appropriate light chains; (ii) high affinity for VRC01-class bNAbs to provide an affinity gradient to guide early somatic mutation toward

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the mature bNAbs. Furthermore, we developed self-assembling nanoparticles presenting 60 copies of the germline-targeting eOD to enhance B cell activation and to improve trafficking to lymph nodes (Fig. 1).

Engineering and Biophysical Analysis of Germline-Targeting Antigens

Modifications to the VRC01 epitope included removing clashes and building new contacts between the CD4bs and the GL-Abs, as well as rigidifying the CD4bs in a conformation that is favorable for binding. Initially, we constructed a homology model of GL-VRC01 bound to gp120 and identified a likely clash between CDRL1 and the N276 glycan. Therefore, we evaluated the GL-VRC01 binding of an eOD (eOD-Base) that lacks glycans at 276 and on the nearby V5 loop owing to N276D and N463D mutations. The eOD-Base barely interacted with GL-VRC01 ($K_D \sim 1$ mM) and had low affinity for only two of eight other GL VH1-2*02 Abs tested (Table 1). We then used Rosetta computational protein interface design (18) to identify other mutations in and around the CD4bs that were predicted to improve GL-VRC01 binding and created directed libraries that included all possible combinations of the computationally identified mutations. These libraries were screened for gp120 core and eOD variants that showed GL-VRC01 binding using yeast cell surface display (19). This strategy gen-

erated germline-targeting (GT) variants of gp120 core (CoreBal-GT1) and eOD (eOD-GT1) that bound GL-VRC01 with K_{DS} of 1.8 μ M and 44 μ M, respectively (Table 1 and table S2). We focused further development on the smaller eOD because it lacks potentially distracting epitopes on gp120 core. A second round of computational design and directed-library screening produced eOD-GT2 with a three-fold improvement in K_D for GL-VRC01 (table S2). Subsequent screening of mixed computational/random mutagenesis libraries led to larger improvements and resulted in eOD-GT3 and eOD-GT4, which had K_{DS} for GL-VRC01 of 220 and 34 nM, respectively (table S2). Interestingly, screening for GL-VRC01 binding also improved binding to other GL VH1-2 Abs, as eOD-GT4 bound to GL-NIH45-46 and GL-PGV19 with K_{DS} of 1.0 μ M and 28 nM, respectively (table S2). To achieve these improvements, eOD-GT4 had accumulated 17 mutations relative to eOD-Base.

To retain as native a CD4bs as possible (by reducing the number of mutations) while also maintaining or improving binding to GL VH1-2 Abs and mature bNAbs, we employed multi-target optimization. Here, libraries with either the wild-type HIV-1 strain HxB2 residue or the mutation in eOD-GT4 were sorted in parallel against multiple Abs, and mutations were retained only if they were positively selected by at least one GL Ab and not negatively selected by other Abs used during optimization. eOD-GT6

was generated by sorting against GL Abs for VRC01, NIH45-46, PGV19, PGV04, and VRC-CH31, as well as mature VRC01 and PGV04. eOD-GT6 had only eight mutations relative to eOD-Base (10 mutations relative to HxB2 gp120) and retained excellent binding to diverse GL VH1-2*02 Abs, with a K_D of 44 nM for GL-VRC01 and $K_{DS} < 500$ nM for five of nine GL Abs tested (Table 1, table S2, and fig. S3). eOD-GT6 also had high affinity for several mature bNAbs, with K_{DS} of 2, 4, and 88 nM for VRC01, NIH45-46, and PGV19, respectively, and maintained the desired affinity gradient for six of eight Abs tested (Table 1). Further, eOD-GT6 bound with high affinity to GL VRC01-class Abs derived from VH1-2*03 and *04 alleles (tables S3 and S4). eOD-GT6 had no detectable affinity for VH1-2*01, probably due to the absence of Trp^{H50}; however, recent data from sequencing of 1092 human genomes (20) shows that the W50R mutation in VH1-2*01 occurs at a frequency of 0.21, indicating that only ~4% of the population are VH1-2*01 homozygotes not amenable to eOD-GT6 priming.

Crystallographic Analysis

To understand the molecular interaction of eOD-GT6 with GL-VRC01, we solved three crystal structures: unliganded GL-VRC01, unliganded eOD-GT6, and the complex of GL-VRC01 bound to eOD-GT6, to resolutions of 2.1 Å, 2.5 Å, and

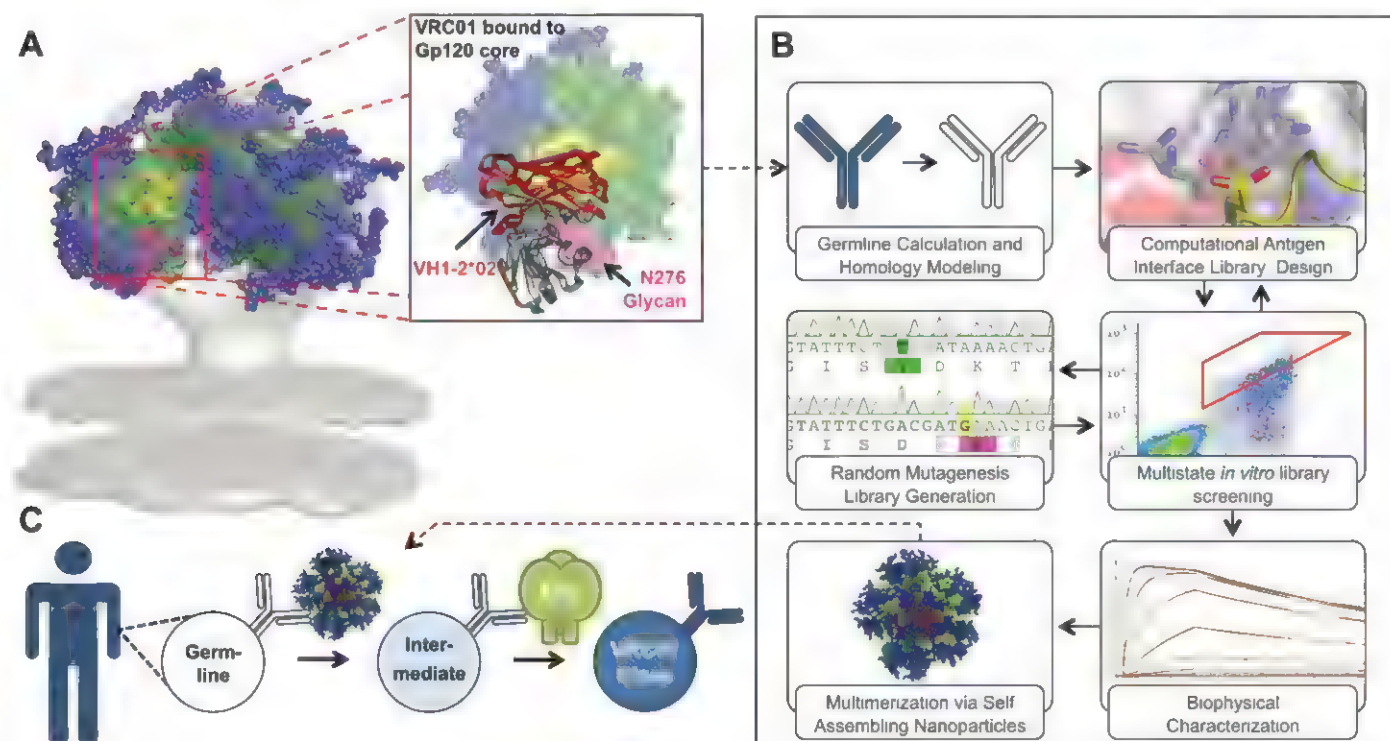


Fig. 1. Development of a germline (GL)-targeted HIV immunogen. (A) VRC01-class bNAbs bind to gp120 primarily through paratope residues encoded by VH 1-2*02. gp120 is colored green, with the CD4 binding site highlighted in yellow. Glycans are represented as blue spheres with the critical N276 highlighted in magenta. VRC01 is shown as a secondary structure rendering and

colored gray, with the VH1-2*02 region highlighted in red. **(B)** Steps in the engineering of a modified gp120-based nanoparticle capable of activating GL VRC01-class B cells. **(C)** This nanoparticle can be used in an HIV-1 vaccine GL-prime-boost strategy to bridge this initial recognition gap and initiate clonal expansion and start somatic hypermutation of VRC01-class bNAbs precursors.

2.4 Å, respectively (Fig. 2 and table S4). The unliganded GL-VRC01 structure revealed that the gp120 contacting loops closely resemble those of VRC01 despite extensive affinity maturation of the latter (Fig. 2A and figs. S5 to S7). Unliganded eOD-GT6 showed a similar structure to the outer domains of unliganded and VRC01-bound gp120 core [1.2 Å C α root mean square deviation (RMSD) in both instances] (Fig. 2B and fig. S8), suggesting good mimicry of the CD4bs. The structure of unliganded eOD-GT6 was also similar to eOD-GT6 bound to GL-VRC01 Fab (0.9 Å RMSD), with the largest differences occurring in the flexible loops and in the eOD exit loop (fig. S9). In addition, the structure of the eOD-GT6+GL-VRC01 complex indicated that the VH1-2-encoded domain of GL-VRC01 approaches eOD-GT6 at an angle nearly identical to that of VRC01 with gp120 (Fig. 2C) (4.2° angular difference when the complexes are superposed on the CD4 binding loop). Overall, the buried surface area (BSA) of GL-VRC01 (1076 Å²) on eOD-GT6 (1102 Å²) is nearly identical to VRC01 (1152 Å²) on gp120 core (1120 Å²), further demonstrating the high degree of similarity between the two structures (tables S5 and S6). Key hydrogen-bonding networks are preserved in the GL-VRC01+eOD-GT6 interaction, particularly in the CD4 binding loop (fig. S10). On the other hand, important differences in hydrogen bonds, BSA, and C α positions are observed for interactions in loop D, V5, and the OD exit loop, which contribute to GL-VRC01 reactivity to eOD-GT6 (Fig. 2D and fig. S11).

Mutation Analysis

To understand the affinity contributions of individual mutations, we measured GL-VRC01 binding affinities for point reversions of each mutation (Table 2). Six mutations on eOD-GT6 conferred improved affinity for GL-Abs relative to the starting construct (eOD-Base) that lacked glycans at 276 and 463. The eOD-GT6+GL-VRC01 complex structure revealed that these mutations either are directly involved in the binding interface (T278R, I371F, and N460V) or stabilize loops involved in the interface (L260F, K357R, and G471S) (Fig. 2C). The two mutations with the largest effect on GL-VRC01 binding were G471S and I371F; reversion at these positions reduced GL-VRC01 affinity by factors of 39 and 10, re-

spectively (Table 2). Ser⁴⁷¹, together with Phe³⁷¹ and Phe²⁶⁰, appear to play a role in altering the conformation of the OD exit loop to allow the GL-VRC01 CDRH2 to make H bonds with three additional gp120 residues (G472, G473, and D474) and bury an additional 120 Å² on gp120, resulting in improved binding (Fig. 2, C and D, right inset panel, tables S5 and S6, and fig. S9). Also, the N460V mutation located in V5 improves packing with the antibody and appears to contribute to an altered V5 conformation and pattern of V5 H-bonding with VRC01, as compared with Clade A/E 93TH057 gp120 recognition of VRC01 (Fig. 2D and fig. S12). Reversion of the N460V mutation reduced GL-VRC01 binding by a factor of 2.5 (Table 2).

Removal of key glycosylation sites was necessary for GL affinity. Reintroduction of the N276 glycosylation site in eOD-GT6 (by a double reversion, D276N/R278T) reduced binding by a factor of 140, and the remaining binding was likely due to a small fraction of the eOD-GT6-D276N/R278T that underutilized the N276 glycosylation position (fig. S13). Reversion of R278T alone reduced affinity by a factor of only 3.6 (Table 2). Thus, removal of the 276 glycan appears to release a block on GL-VRC01 binding but does not confer appreciable eOD affinity; further interface modification was required to achieve high affinity. Indeed, the eOD-GT6+GL-VRC01 complex structure revealed that, in addition to removing a clash between the N276 glycan and CDRL1 (Fig. 2C, left inset panel), eOD-GT6 D276 and R278 make two additional H-bonds with GL-VRC01. eOD-GT6 also lacks glycans at positions 386 (B12) and 463 (V5). Restoration of these glycosylation sites reduced affinity for GL-VRC01 by a factor of 3 (table S7).

eOD-GT6 Nanoparticle Generation

To enable eOD-GT6 to activate GL B cells via cross-linking of B cell receptors, and to develop a multivalent platform for eOD-GT6 that mimics the size, shape, multivalency, and symmetric surface geometry of many viruses for improved immunogenicity (21), we sought to fuse eOD-GT6 to a self-assembling virus-like nanoparticle. From a search of large homomeric particles in the Protein Data Bank (PDB), we prioritized 60-member of lumazine synthase from the hyperthermophile *Aquifex aeolicus* for experimental

testing due to their thermal stability and because modeling suggested that, with a suitable linker length, 60 copies of glycosylated eOD-GT6 could be sterically accommodated in an orientation that would expose the VRC01 epitope (Fig. 3A). Although expression of the wild-type particle had been reported in *Escherichia coli* (22), we found that such nanoparticles presenting glycosylated eOD-GT6 could be secreted from mammalian (293) cells and purified by lectin chromatography with good yield (~10 mg/L) and structural homogeneity (Fig. 3B and figs S14 and S15).

In Vitro B Cell Activation

The ability of eOD-GT6 nanoparticles to activate B cells expressing GL and mature VRC01 [immunoglobulin M (IgM)] (23), 12A12 (IgM and IgG), and NIH45-46 (IgG) (24) was tested in Ca²⁺-dependent activation assays. The nanoparticles potentially activated both GL and mature B cells with 1 μM outer domain (16 nM particle) and modestly activated all three cell lines at 1000-fold lower concentrations (Fig. 3C and fig. S16). In contrast, monomeric eOD-GT6 was non-stimulatory, probably due to an inability to cross-link B cell receptors (23). Trimeric eOD-GT6 activated both GL and mature B cells, but less potently and rapidly than the 60-member nanoparticles, and a soluble gp140 trimer from HIV-1 strain YU2 (25) showed no activation of GL B cells but did activate the mature counterparts (Fig. 3C). Both IgM and IgG B cell lines were generated for GL 12A12, and we observed no significant differences in activation magnitude or kinetics between the two antibody isotypes (fig. S16).

Animal Models for Human VH1-2 Germline Targeting

We then assessed whether eOD-GT6 might interact with related GL-Abs in animal models. Analysis of VH genes from rabbit (fig. S17) (26), mouse (figs. S18 and S19) (27), and macaque (fig. S20) revealed that none of these commonly used model organisms have a known VH gene containing all of the critical residues for GL binding (15). To measure binding experimentally, chimeric GL-Abs were produced in which the human VH1-2*02 gene from GL-VRC01 was replaced with GL VH genes from mice or macaques containing the essential Arg^{H71} and as many other

Table 1. Binding of GL and mature (Mat) antibodies to gp120 and eOD variants. Values represent K_Ds in nM measured by surface plasmon resonance (SPR). Detectable binding to GL antibodies is in boldface.

Antigen		Antibody															
		VRC01		12A12		3BNC60		NIH45-46		PGV04		PGV19		PGV20		VRC-CH31	
		GL	Mat	GL	Mat	GL	Mat	GL	Mat	GL	Mat	GL	Mat	GL	Mat	GL	Mat
Wild-type	Core.HXB2	>10 ⁵	5	>10 ⁵	6	>10 ⁵	36	>10 ⁵	35	>10 ⁵	48	>10 ⁵	20	>10 ⁵	19	>10 ⁵	47
Germline-targeted	eOD-Base N276D	>10 ⁵	5	>10 ⁵	380	>10 ⁵	4100	>10 ⁵	14	>10 ⁵	110	>10 ⁵	3100	17,000	16	>10 ⁵	30,000
	Core.BaL-GT1	1800	0.5	3200	1	16,400	4	>10 ⁵	0.6	>10 ⁵	170	25	14	5,500	4	35,000	1800
	eOD-GT1	44,000	1	>10 ⁵	2300	>10 ⁵	83	>10 ⁵	3	>10 ⁵	4	7800	1000	1100	10	>10 ⁵	>10 ⁵
	eOD-GT6	44	2	2000	400	14,000	200	410	4	52,000	10	19	88	3	6	28,000	29,000

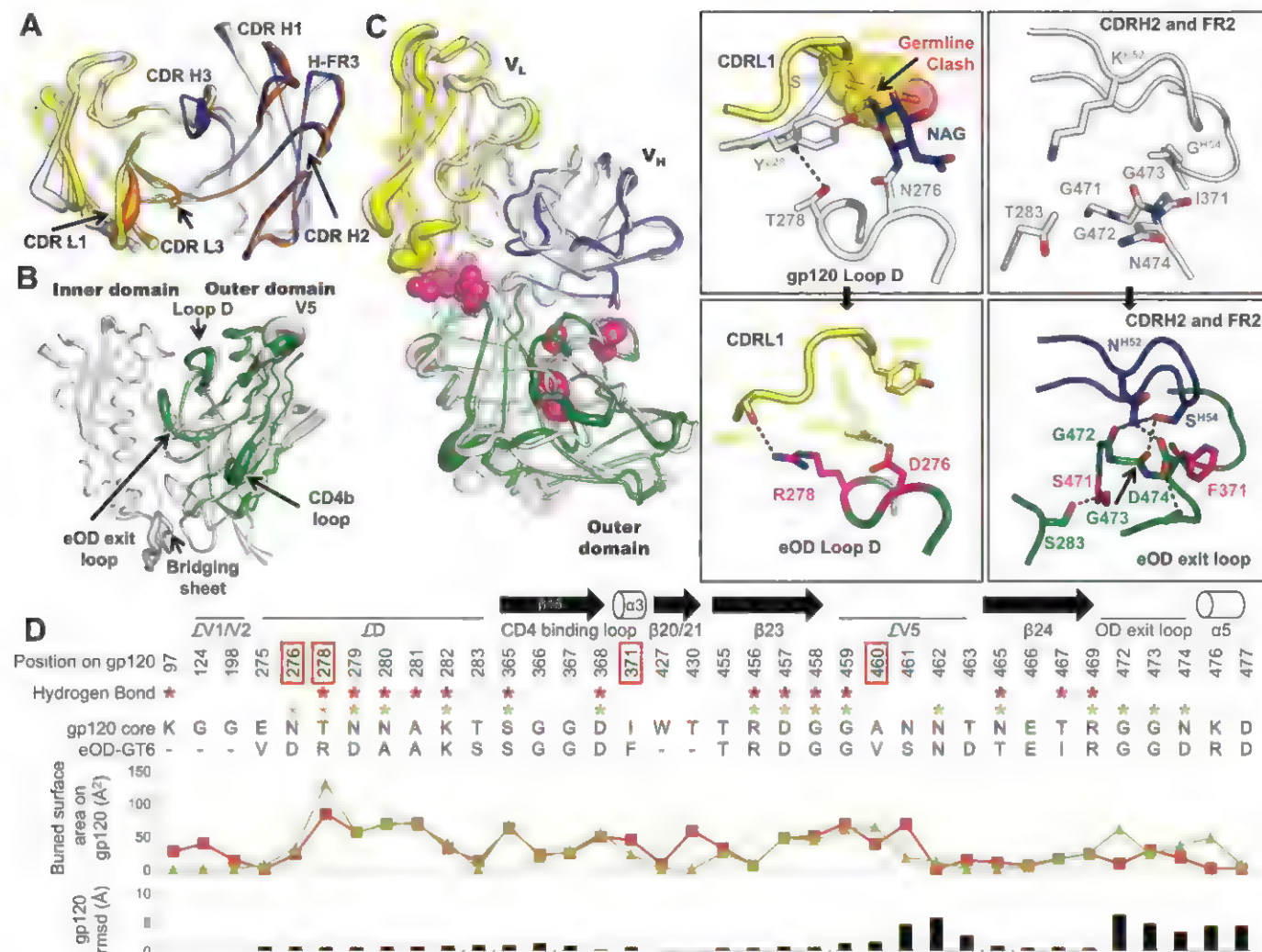


Fig. 2. Structural analysis of GL-VRC01 and eOD-GT6. (A) The structure of the unliganded inferred GL-VRC01 antibody (heavy and light chains colored blue and yellow, respectively) is similar to the structure of gp120-bound VRC01 (white) within the gp120-contacting positions (shown in orange on VRC01, defined by the structure of VRC01+gp120 in PDBID: 3NGB). Structures are rendered according to B values, with thin and thick lines representing areas possessing low and high flexibility, respectively. (B) Comparison between the crystal structures of unliganded eOD-GT6 (green) and unliganded gp120 core from HIV-1 strain 93TH057 (PDBID: 3TGT, white). Structures rendered as in (A). (C) Comparison between the crystal structures of GL-VRC01+eOD-GT6 and VRC01+gp120 core (PDBID: 3NGB), in which only the outer domain of gp120 is shown. Structures rendered as in (A) and (B), except gp120-contacting positions on VRC01 are white. The mutated residues in eOD-GT6 that enable

binding of GL-VRC01-class Abs are shown as space-fill magenta spheres. The angle of approach of GL-VRC01 and VRC01 to the CD4bs is nearly identical. Key regions where interactions are different between VRC01 on gp120 (upper panels) and GL-VRC01 on eOD-GT6 (lower panels) are shown in insets. eOD-GT6 confers germline reactivity by removing a potential clash with the N276 glycan, as well as by creating additional contacts with loop D (left panels), the OD exit loop (right panels) and V5 (fig. S12). (D) gp120 residues involved in the VRC01+gp120 and GL-VRC01+eOD-GT6 interfaces are compared in sequence, hydrogen bonding (stars), buried surface area, and RMSD. Interfaces were calculated using PDBePISA (29) and C α RMSD using Chimera (30). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

critical residues as possible. Chimeric GL-Abs with mouse VH genes had no detectable binding to eOD-GT6. Chimeric Abs derived from two of the three rhesus VH genes bound weakly to eOD-GT6, with K_D s of ~30 μ M and ~40 μ M (table S8). The rhesus chimeric GL-Ab most similar to human GL-VRC01 contained only 10 mutations in the VH gene (95.5% identity over the antibody Fv region) but showed no detectable binding to eOD-GT6. Annotation of the rhesus macaque antibody repertoire and analysis of gene usage frequencies will be useful to construct bona fide macaque GL VH1-2 Abs. These

analyses illustrate the potential difficulty for using animal models to produce VRC01-class bNAbs and suggest that immunization of humans or mice engineered to produce human Abs may be essential for testing and iteratively optimizing such immunogens.

Concluding Remarks

The events that led to GL VH1-2*02 B cell activation in the HIV-infected individuals from which VRC01-class bNAbs were isolated remain unclear. Our finding that a small number of rare or previously undocumented Env mutations con-

fers high affinity GL binding suggests that Env variants might have acquired one or more such mutations stochastically during infection and thereby gained the ability to prime GL VRC01-class B cells. Vaccines to induce VRC01-class responses will need to activate such B cells dependably and drive appropriate somatic mutation to produce high-affinity bNAbs (28). We propose the eOD-GT6 nanoparticle as a promising candidate for a vaccine prime based on its ability to bind diverse VH1-2*02 GL Abs, activate VRC01, 12A12, and NIH45-46 B cell lines in vitro, and provide an affinity gradient for early

Table 2. Binding of GL VRC01 to eOD-GT6 point reversions. Values represent K_D s in nM measured by SPR. Amino acid frequencies determined from 2867 HIV-1 sequences from www.hiv.lanl.gov. epPCR, error-prone polymerase chain reaction.

	eOD-GT6	Single reversions					
Reversion from eOD-GT6	—	F260L	R278T	R357K	F371I	V460N	S471G
Frequency of WT amino acids	—	L (97.3%)	T (78.4%)	K (68.9%)	I (85.8%)	N (36.2%)	G (78.7%)
Frequency of mutation	—	F (0.0%)	R (0.1%)	R (0.5%)	F (0.0%)	V (3.4%)	S (0.5%)
Method of discovery	—	epPCR	Rosetta	epPCR	Rosetta	Rosetta	epPCR
GL-VRC01 affinity (nM)	44	310	160	64	450	120	1,700
Change from eOD-GT6	—	7.0	3.6	1.5	10.3	2.7	39

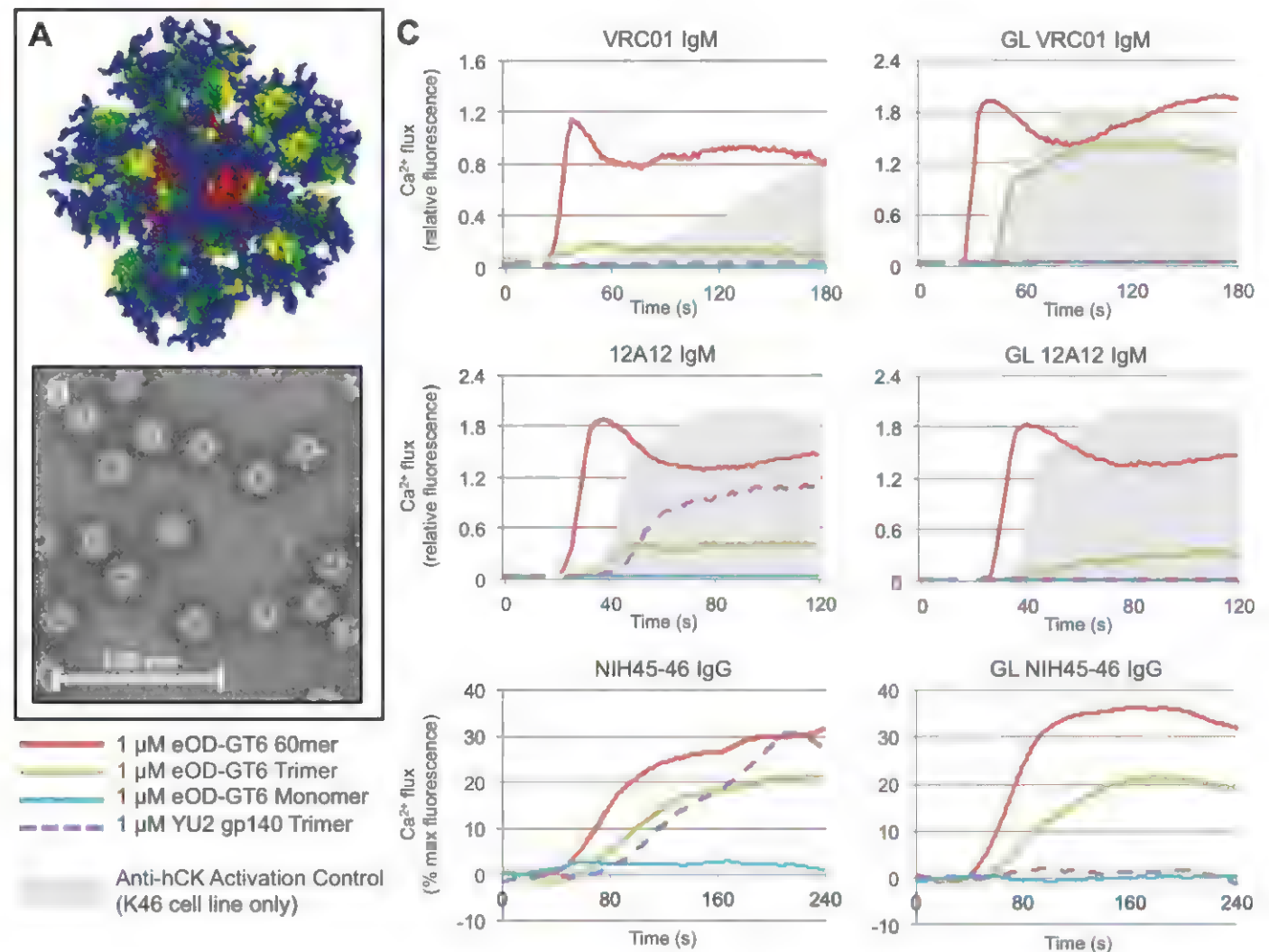


Fig. 3. A 60-member eOD-GT6 nanoparticle activates GL and mature VRC01-class B cells. (A) Model representation of the 60-member eOD-GT6 nanoparticle. eOD-GT6 is colored in green, with residues that interact with VRC01 colored in yellow. Glycans are shown as blue spheres, and the self-assembling 60-member lumazine synthase to which eOD-GT6 is fused is colored red. (B) Raw negative stain electron microscopy image of the 60-member

eOD-GT6 nanoparticle. (C) Calcium flux experiments show that the 60-member eOD-GT6 nanoparticle activates B cell lines engineered to express either GL or mature VRC01 IgM, 12A12 IgM, or NIH45-46 IgG, whereas a recombinant soluble gp140 trimer activates the B cells expressing mature, but not GL VRC01-class, antibodies. Data for each antibody are representative of at least two separate experiments.

somatic mutation. We further propose that ultimate elicitation of mature VRC01-class bNAbs will require, at minimum, boosting with different immunogens that present a less engineered, more native CD4bs, including the glycans around the CD4bs.

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REPORTS

Lorentz Meets Fano in Spectral Line Shapes: A Universal Phase and Its Laser Control

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Symmetric Lorentzian and asymmetric Fano line shapes are fundamental spectroscopic signatures that quantify the structural and dynamical properties of nuclei, atoms, molecules, and solids. This study introduces a universal temporal-phase formalism, mapping the Fano asymmetry parameter q to a phase φ of the time-dependent dipole response function. The formalism is confirmed experimentally by laser-transforming Fano absorption lines of autoionizing helium into Lorentzian lines after attosecond-pulsed excitation. We also demonstrate the inverse, the transformation of a naturally Lorentzian line into a Fano profile. A further application of this formalism uses quantum-phase control to amplify extreme-ultraviolet light resonantly interacting with He atoms. The quantum phase of excited states and its response to interactions can thus be extracted from line-shape analysis, with applications in many branches of spectroscopy.

In spectroscopic detection of electromagnetic radiation, the sample's temporal dipole response—the time-dependent dipole moment of the system after an infinitesimally short (Dirac delta function) excitation—gives rise to line shapes observed in fluorescence or absorption. If a continuum of states is excited, this temporal

dipole response corresponds also to a delta function, which is the superposition of a continuous spectrum of emitting dipoles at all frequencies. The more commonly observed exponential decay of a discrete excited state with a finite lifetime gives rise to the well-known symmetric Lorentzian line shape.

Asymmetric Fano absorption line shapes emerge when discrete excited states are coupled to a continuum of states (1, 2), which is a general phenomenon throughout nuclear (3), atomic (4–6), and solid-state physics (7–10), as well as molecular spectroscopy in chemistry (11). As a result of this discrete-continuum coupling mech-

anism, the temporal dipole response function is not just the sum of the exponentially decaying and delta-like dipole responses of the isolated state and continuum, respectively. The exponential dipole response is shifted in phase with respect to the Lorentzian response, which is the origin of the asymmetric line shape of the Fano resonance. By a mathematical transformation [supplementary text (12) section 1] similar to the one recently conducted for a classical Fano oscillator (13), we mapped this phase shift φ in the time domain into the q parameter, which was introduced by Ugo Fano (1, 2) and thereafter used to characterize and quantify the asymmetric Fano line shape. The cross section at photon energy $E = \hbar\omega$ is given in terms of q by

$$\sigma_{\text{Fano}}(E) = \sigma_0 \frac{(q + \epsilon)^2}{1 + \epsilon^2}, \epsilon = \frac{E - E_0}{\hbar\Gamma/2} \quad (1)$$

where ϵ denotes the reduced energy containing E_0 and Γ as the position and width of the resonance, respectively, $\hbar$ denotes the reduced Planck constant, and σ_0 is the cross section far away from the resonance.

In general, the absorption cross section σ_{abs} is proportional to the imaginary part of the index of refraction, which in turn is directly related to the polarizability (5) and thus to the frequency-dependent dipole response function $d(E)$:

$$\sigma_{\text{abs}}(E) \propto \text{Im}[d(E)] \quad (2)$$

Via the Fourier transform, $d(E)$ is connected to the time-dependent linear response $\tilde{d}(t)$ of the medium after a delta-like excitation pulse. For a Lorentzian spectral line shape of width Γ , $\tilde{d}_{\text{Lorentz}}(t)$ is an exponentially decaying function

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of time with time constant $\tau = 1/\Gamma$. For the Fano resonance, equating $\sigma_{\text{abs}} = \sigma_{\text{Fano}}$ (Eqs. 1 and 2) and using causality results in the following expression for the time-dependent response function [see supplementary text (12) section 1 for the derivation]

$$\tilde{d}_{\text{Fano}}(t) \propto c_q \delta(t) + \exp\left\{-\frac{\Gamma}{2}t + i\left[-\frac{E_0 t}{\hbar} + \varphi(q)\right]\right\} \quad (3)$$

that is the sum of a scaled Dirac delta function $\delta(t)$ (with scaling factor c_q) and an exponentially decaying dipole moment with a phase given by

$$\varphi(q) = 2\arg(q - i) \quad (4a)$$

and in turn

$$q(\varphi) = i \left[\frac{1 + \exp(i\varphi)}{1 - \exp(i\varphi)} \right] = -\cot\left(\frac{\varphi}{2}\right) \quad (4b)$$

For the special case of $\varphi = 0 = c_q$, the Lorentzian dipole response function is obtained. This correspondence is illustrated in Figs. 1 and 2.

In the context of configuration interaction of electronic states as considered in Fano's original theory, this phase φ can be understood as the con-

sequence of the strong coupling of continuum and discrete quantum states. Originally this coupling was formulated in terms of the q parameter as shown in Eq. 1. The reformulation here provides a physically intuitive picture in terms of the dipole response function and unveils the universal nature of the phase φ . In particular, an important implication of this mapping between q and the phase φ is the fact that any phenomenon that shifts the dipole evolution of a system out of phase with an initial excitation can be used to modify the q parameter—for example, transforming a Lorentzian into a Fano line-shape profile (14) and vice versa—and thus to control the absorption process in general.

Since their invention, laser light sources have evolved technologically to provide few-cycle light fields in the visible and near-infrared (NIR) and delta-like attosecond pulses (15) in the extreme ultraviolet (XUV) spectral regions. Having these optical precision tools at hand, here we ask the scientific questions: Can we use lasers to quantum-simulate Fano resonances? To what extent can absorption, in general, be controlled?

Answers to these questions could help to understand and to formulate new descriptions of electron-electron interaction dynamics on one hand and enable x-ray or even γ -ray laser applications on the other hand.

In the following, we start out by experimentally proving and applying the above Fano- q /dipole-phase correspondence to the paradigmatic two-electron system of helium (to which Fano's theory was originally applied in his 1961 formulation), switching a Fano line shape into a Lorentzian line shape and then vice versa by introducing an additional phase shift with a laser field.

In the experiment, broadband attosecond-pulsed XUV light [few-pulse train; see supplementary text (12) section 3 for the experimental setup] was sent through a sample of He atoms at a density of ~ 100 mbar, serving as a delta-like excitation compared to the state lifetime. The transmitted spectrum (exciting XUV pulse plus coherent dipole response) was resolved by a flat-field grating spectrometer in the vicinity of the sp_{2n+} doubly excited state resonance series and detected with a backside-illuminated x-ray charge-coupled device camera. In addition, a collinearly propagating 7-fs NIR laser pulse of controlled intensity was directed through the sample at a fixed delay of ~ 5 fs after the XUV pulse. The intensity of the NIR pulse was controlled by opening and closing an iris in the beam path. When the NIR pulse was absent, the coherent dipole response produced the natural absorption spectrum; that is, the commonly known Fano line shapes were observed (Fig. 3A). With a laser field present, the coherent dipole response was modified ~ 5 fs after its excitation, and we observed strong modifications of the spectral features (16). At a laser intensity of $2.0 \times 10^{12} \text{ W/cm}^2 \pm 1.0 \times 10^{12} \text{ W/cm}^2$, the asymmetric Fano profiles were replaced by symmetric Lorentzian line shapes in the spectrum (Fig. 3B). Assuming a ponderomotive shift of these states in the laser field during the pulse duration [supplementary text (12) section 2], we calculated a total phase shift of $(-0.35 \pm 0.18)\pi = -1.1 \text{ rad} \pm 0.5 \text{ rad}$ imprinted by the NIR pulse right after (i.e., short compared to the state lifetime) the excitation. From Eq. 4, the dipole phase φ of the original Fano resonance sp_{24+} , exhibiting a q of -2.55 (6), amounts to $\varphi = 0.24\pi$ (0.75 rad). The two phases add up, and the NIR pulse thus compensates the original (Fano) dipole phase of the state and shifts it back to near zero $(-0.11 \pm 0.16)\pi$ (Fig. 2), producing the Lorentzian absorption line (Fig. 3B). The lifetime of the considered states, is on the order of 100 fs or longer and not affected by the much shorter NIR pulse. In this limit, the phase modification right after excitation can be considered kicklike (impulsive). We can thus apply the above (Eq. 4) mapping between φ and q as a good approximation. Note that all states shown in Fig. 3A exhibit $q = -2.55 \pm 0.03$. The fact that all of these states assume their Lorentzian profile (Fig. 3B) at the same intensity is thus a further confirmation of the validity

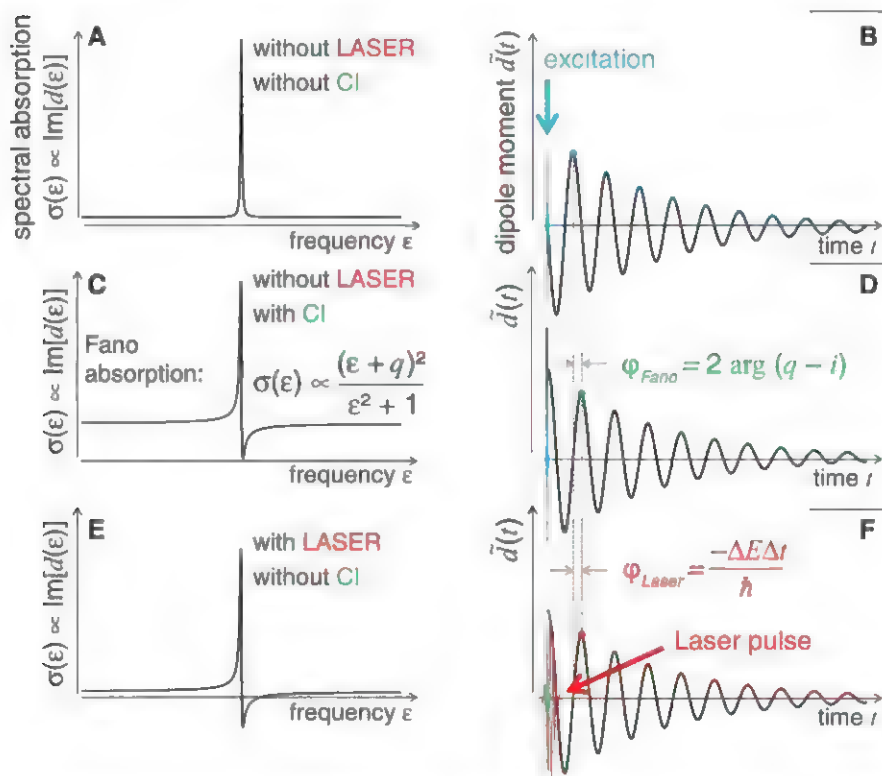


Fig. 1. Lorentz and Fano line shapes, their temporal dipole response functions, and a universal phase that governs configuration interaction and pulsed (kicklike) laser interaction with a state. (A) Lorentzian spectral absorption line shape. (B) Lorentzian temporal dipole response function, exhibiting the well-known exponential decay. (C) Fano spectral absorption line shape. (D) Fano temporal dipole response function, consisting of an instantaneous delta function (continuum absorption) and an exponentially decaying response, governed by autoionization. The asymmetric line shape is understood to be caused by the phase shift φ_{Fano} of the long-lived dipole response as compared to the Lorentzian case. This phase shift is created by configuration interaction (CI) associated with electron-electron interaction in the case of a traditional Fano resonance. (E and F) Alternatively, a Lorentzian decaying state can be laser-dressed impulsively (kicklike) immediately after its excitation, inducing a phase shift φ_{Laser} governed by the product of duration of the laser pulse Δt and energy shift ΔE . The state will then decay with a phase shift of its dipole moment (F), inducing the same Fano-like absorption line shape (E).

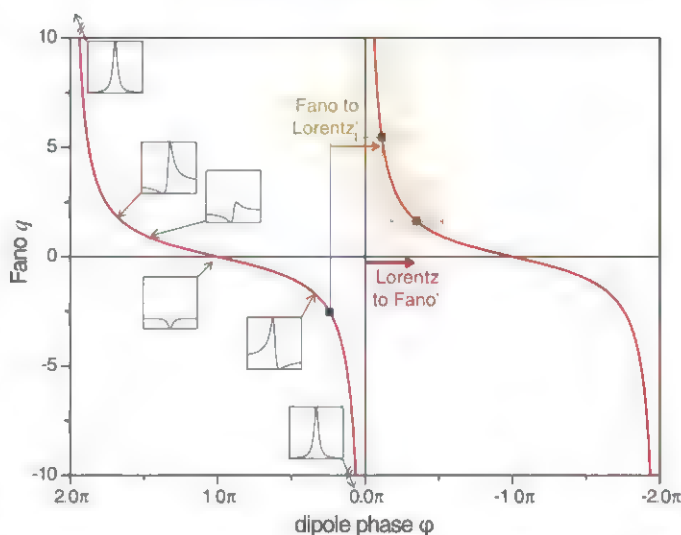
of the introduced formalism. The lower-lying states, such as the $2s2p$ and sp_{23+} , exhibit a complicated mixture of the here-discussed nonresonant (ponderomotive) and resonant coupling via the $2p^2$ state (16). This results in more complicated and time-dependent dipole phase and amplitude shifts beyond the applicability of the present formalism.

To provide additional evidence for the validity of this concept, we experimentally applied the time-domain Fano-phase framework to singly excited states in He. For these states, their natural decay proceeds fully radiatively with no interfering continuum and thus normally results in Lorentzian absorption line shapes (17). Here, we show the inverse transformation to the case above: the trans-

formation of an initially Lorentzian atomic absorption line into a Fano resonance.

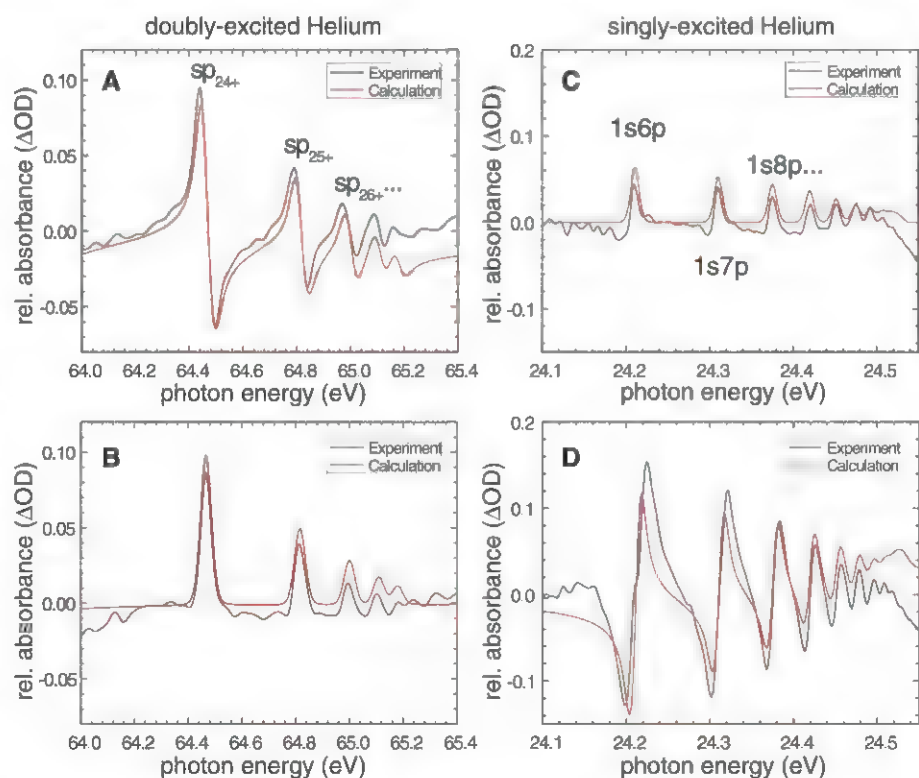
We used the same experimental configuration as above, except with the spectrometer set to resolve lower photon energies in the vicinity of 24 eV. When the laser field was absent, we recorded the well-known absorption spectrum of helium in the $1snp$ series just below its first ionization threshold at 24.6 eV (Fig. 3C). When the laser intensity was set to $2.1 \times 10^{12} \text{ W/cm}^2 \pm 1.1 \times 10^{12} \text{ W/cm}^2$, the symmetric Lorentzian line shapes were replaced by asymmetric Fano line shapes (Fig. 3D). Again, calculating the ponderomotive phase shift induced by the laser at this intensity results in $\varphi = (-0.38 \pm 0.19)\pi = -1.2 \text{ rad} \pm 0.6 \text{ rad}$. According to the time-domain phase formalism (Eq. 4), this implies a Fano q of +1.49 with experimental error as indicated by the red shaded area in Fig. 2, which is in agreement with the measured line shape in Fig. 3D. The line broadening in Fig. 3D is caused by a reduction in the states' (originally nanosecond) life times resulting from single-photon ionization in the long-duration pedestals typically accompanying few-cycle laser pulses. Small additional line-shape modifications on the $1s6p$ and lower-lying states are due to the onset of near-resonant couplings to other bound states. Their description is currently beyond the scope of the phase-shift model introduced here. A sub-cycle time-delay-dependent shift of the $1s3p$ and $1s4p$ resonances has recently been observed in transient-absorption measurements (18), whereas here we focus on a global change of the dipole

Fig. 2. Mapping of Fano's q (line-shape asymmetry) parameter to the temporal response-function phase φ . A bijective map between the two parameters is obtained in a range $[-\pi, \pi]$, while the function is periodic in 2π . Lorentzian line shapes are obtained for the extreme cases of $\varphi \rightarrow 2m\pi$ (integer n), corresponding to $q \rightarrow \infty$ and $q \rightarrow -\infty$, respectively, whereas between these regimes Fano line shapes are obtained, with the special case of a window resonance at $\varphi = (2n + 1)\pi$, $q = 0$. (Insets) The absorption line shapes $\sigma(\epsilon)$ (as depicted in Fig. 1) for selected values of $q(\varphi)$. The laser interaction creates an additional phase shift (horizontal arrows) that changes the character of the observed resonance line shape. The dots represent the situations measured in the experiment and shown in Fig. 3. The shaded areas represent the errors which are given by the experimental uncertainty in the intensity determination.



formation of an initially Lorentzian atomic absorption line into a Fano resonance.

Fig. 3. Transforming asymmetric Fano spectral absorption lines into symmetric Lorentzian absorption peaks in doubly excited He and vice versa, from Lorentz to Fano, in singly excited He. (A) Field-free (static) absorption spectrum of doubly excited states of the $N = 2$ series in He. The well-known Fano absorption profiles are observed in the transmitted spectrum of a broad-band attosecond pulse. (B) When a 7-fs laser pulse immediately follows the attosecond-pulsed (deltalike) excitation (time delayed by ~ 5 fs) at an intensity of $2.0 \times 10^{12} \text{ W/cm}^2$, the Fano absorption profiles are converted to Lorentzian profiles. (C) Field-free (static) absorption spectrum of singly excited He states below the first ionization threshold (24.6 eV): Lorentzian line shapes are visible in the attosecond-pulse absorption spectrum. (D) Absorption spectrum of the states in (C), when the attosecond pulse is again followed by the 7-fs laser pulse, at an intensity of $2.1 \times 10^{12} \text{ W/cm}^2$. The initially Lorentzian absorption profile has been laser-transformed into an asymmetric Fano profile. The solid black lines are the measurement results; the red lines are generated by using tabulated values in (A) from (6) and (C) from (30), whereas the red line in (B) represents Lorentzians at the resonance positions of the original Fano lines. The red line in (D) shows Fano profiles with expected laser-induced $q = 1.49$ (Fig. 2) at the resonance positions of the original Lorentzian resonances. [For details of the calculated profiles and the experimental data, see supplementary text (12) sections 4 and 5, respectively.] ΔOD , relative optical density as defined in supplementary text (12) section 5.



phase φ right after excitation (at a constant time delay) and relate it to the transformation of the Fano q parameter.

In addition to the change of the line profile, we can also control the sign of the absorption by varying only the phase φ . Thus, gain can be optically induced solely by modifying the phase of the polarization response after perturbative excitation in the absence of additional amplitude control or even population inversion of the excited state. This becomes clear by starting out from the dipole response of a Lorentzian absorption line (with an unperturbed $\varphi = 0$), where the laser-controlled tunable phase φ is again introduced to obtain

$$\tilde{d}(t) \propto \exp\left[-\frac{\Gamma}{2}t + i\left(-\frac{E_0 t}{\hbar} + \varphi\right)\right] \quad (5)$$

resulting in

$$\begin{aligned} \sigma_{\text{abs}}(E) &\propto \text{Im}[d(E)] \\ &= \text{Im}\left(-\frac{\varepsilon}{1+\varepsilon^2}e^{i\varphi} + i\frac{1}{1+\varepsilon^2}e^{i\varphi}\right) \text{ with} \\ \varepsilon &= \frac{E-E_0}{\hbar(\Gamma/2)} \end{aligned} \quad (6)$$

which now can become negative when φ departs from its original (no laser) value of 0.

This is because the negatively valued (for $\varepsilon < 0$) dispersive term $\frac{\varepsilon}{1+\varepsilon^2}$ rotates, in the complex plane, by the angle φ and acquires an imaginary part. The phase φ can thus be interpreted as a laser-controllable mixing angle between dispersion [the real part of $d(E)$] and absorption.

To confirm this control and transformation of absorption into gain, we acquired a spectrum spatially resolved across the vertical axis, with and without laser control (Fig. 4). The presence of the laser field enhances the EUV light intensity at the resonance positions of the singly excited states of helium, which are normally absorbing (in the absence of the laser field). The same general mechanism could, in the future, be applied to hard-x-ray or even γ -ray transitions with much longer (>100 ns, for example, ^{57}Fe Mössbauer) life times. There, the $\varphi = \pi$ phase flip could also be achieved, for example, by nanosecond-pulsed magnetic fields (Zeeman shift) or a physical displacement of a solid-state absorber (19) right after excitation by a femtosecond free-electron laser (FEL) or a subnanosecond synchrotron pulse.

Having this experimental confirmation, Eq. 6 thus allows a general interpretation of effects such as electromagnetically induced transparency (EIT)

(20, 21) $\{\sigma_{\text{abs}}(E) \propto \text{Im}[d(E)] = 0\}$ and lasing without inversion (22, 23) $\{\sigma_{\text{abs}}(E) < 0\}$ in a unified intuitive picture. It also connects to general dispersion control (14, 23) with short-pulsed fields. The formalism developed here is universal and may also help to provide intuitive physical pictures for the case of nonresonant amplification of light recently experimentally observed in helium atoms (24). The Fano-phase formalism provides a time-domain picture for existing theory (25) and experiments on the laser coupling and control of Fano resonances (26), including recent work using attosecond pulses (27, 28). In a complementary theoretical work, modifications of Fano profiles in transient-absorption spectra for the case of two laser-coupled Fano resonances (including coherent population transfer) have recently been observed and analytically described (29). Going beyond the approximation of the kicklike phase shift near time zero as discussed here could provide enhanced physical understanding of such general time-delay-dependent transient-absorption measurements.

The existence of a direct correspondence between Fano's q parameter and the dipole phase of an excited state has thus been proved. Because the latter is susceptible to laser fields, Fano absorption profiles can be induced in the absence of effects such as autoionization. This is important because the change of the absorption profile is in turn a measure of the induced phase shift of a complex quantum-mechanical state amplitude in a laser field, with numerous applications in spectroscopy and quantum-state holography. The now well-understood phase-to- q correspondence allows mapping of the coupling of these states to laser fields or other interactions, providing information especially when the coupling mechanisms are more complex than just a ponderomotive coupling, which was considered here to introduce the principle. These additional couplings are already expected in helium atoms for the more strongly bound quantum states, or for states in which both electrons are excited to the same or similar quantum numbers, for which electron-electron interaction effects play a major role while interacting with the laser. There is no reason why the mechanism should not be applicable to molecules or excitons in condensed phase or mesoscopic materials. The Fano-phase formalism also provides an intuitive link between quantum (configuration interaction, energy shifts of quantum states) and classical phenomena (classical light fields, phase shifts of oscillators), which could possibly spawn novel quantum-classical hybrid pictures of multielectron dynamics.

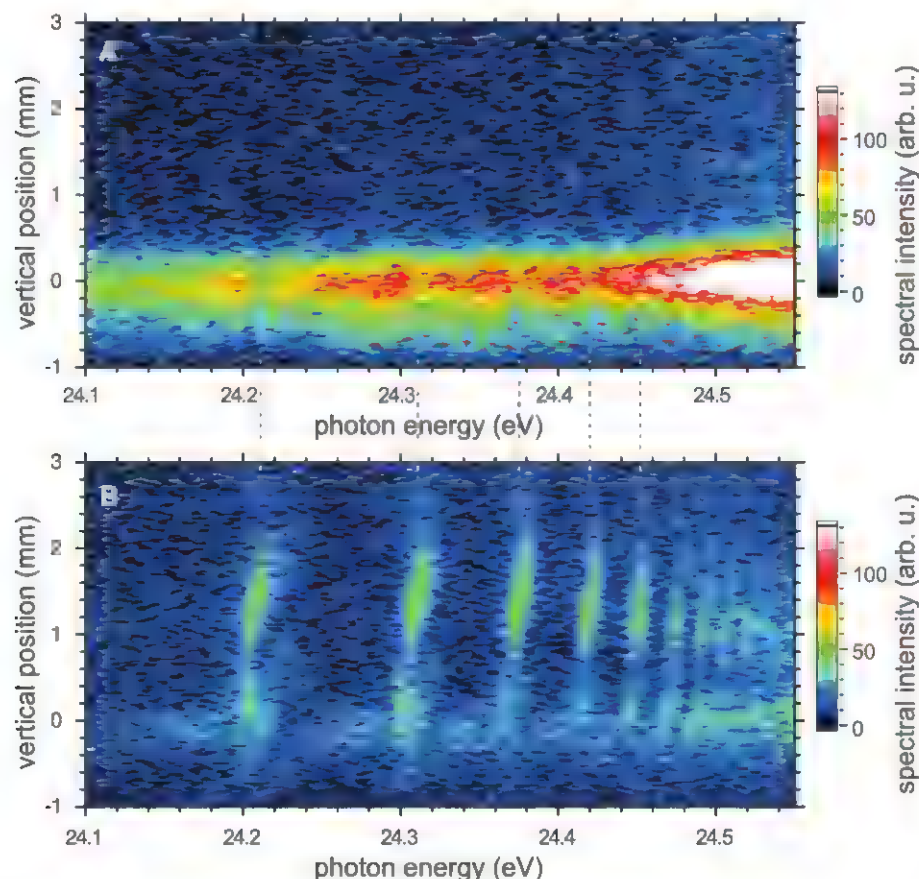


Fig. 4. Laser-controlled amplification of resonant light in the EUV. (A) Spectrum of transmitted EUV light without control laser: The helium resonant absorption lines can be observed as local minima in an otherwise smoothly varying spectrum centered at a vertical position of 0 mm. **(B)** Spectrum of transmitted and amplified EUV light in the presence of the control laser. Amplification can be observed exactly at the He resonance positions corresponding to absorption in (A). It is also observed slightly off-axis, which is likely due to imperfect angular alignment of the optical control laser with respect to the EUV beam.

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Supplementary Materials

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Supplementary Text
Figs. S1 to S3
Tables S1 and S2
References (30–34)

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Multiscale Modeling of Membrane Rearrangement, Drainage, and Rupture in Evolving Foams

Robert I. Saye¹ and James A. Sethian^{1*}

Modeling the physics of foams and foamlike materials, such as soapy froths, fire retardants, and lightweight crash-absorbent structures, presents challenges, because of the vastly different time and space scales involved. By separating and coupling these disparate scales, we have designed a multiscale framework to model dry foam dynamics. This leads to a predictive and flexible computational methodology linking, with a few simplifying assumptions, foam drainage, rupture, and topological rearrangement, to coupled interface-fluid motion under surface tension, gravity, and incompressible fluid dynamics. Our computed results match theoretical analyses and experimentally observed physical effects, including thin-film drainage and interference, and are used to study bubble rupture cascades and macroscopic rearrangement. The developed multiscale model allows quantitative computation of complex foam evolution phenomena.

One of the hallmarks of natural phenomena is that they often occur across multiple space and time scales. An example comes from climate studies, in which oceanic and atmospheric waves spanning hundreds of kilometers are influenced by temperature variations in localized environments. In such “multiscale problems,” the unfolding of small-scale processes, depending on physics, chemistry, and biology, combine to produce large-scale effects, and these macroscopic dynamics subsequently affect the interplay of microscopic forces. Traditionally, computational models rely on accurately modeling the smallest possible space and time scales. However, in a multiscale problem, this technique may require such a fine resolution that there is no practical hope of following a calculation long enough to observe the macroscale behavior, even with today’s advanced computing hardware.

Fortunately, the details at one space or time scale are not necessarily important at another scale. By devising different models and equations at different scales, we can “separate scales” and compute physics at different resolutions, allowing these different models to communicate across

the scales. The challenge is to find such a scale separation and develop models that are computationally tractable, so that critical information is communicated between scales without creating artificial physics.

An everyday example of multiscale physics can be found in foams, which have a wide variety of applications in industry and materials design. Liquid foams, characterized by fluid-filled mem-

branes separating gaseous regions, include soapy detergents, substances to separate out hydrophobic molecules, and even the head on a beer. Solid foams, formed by solidifying liquid foams, include lightweight materials such as metallic and plastic foams. Understanding the dynamics of foam evolution is a key step in controlling the structure and properties of foamlike materials. Deriving models to quantitatively predict foam evolution is challenging because the underlying physics takes place over vastly different time and space scales.

In this work, we use a foam of common soap bubbles as our prototypical example. A single bubble consists of a thin membrane of fluid, known as the lamella, separating the inside gas from the outside. In a cluster of such bubbles (Fig. 1A), multiple lamellae meet at junctions called Plateau borders, forming a network of interconnected thin-film membranes. The dynamics of this foam cluster are intricate (1) and depend on a complex interaction between microscale fluid flow inside the lamellae and Plateau borders, and the macroscale motion of the gas inside the bubbles. To illustrate, consider a foam whose macroscopic configuration appears to be in equilibrium, such as the foam in Fig. 1A. Although seemingly stable, liquid inside the films drains over time, owing to effects of gravity and surfactant. When one of the membranes becomes too

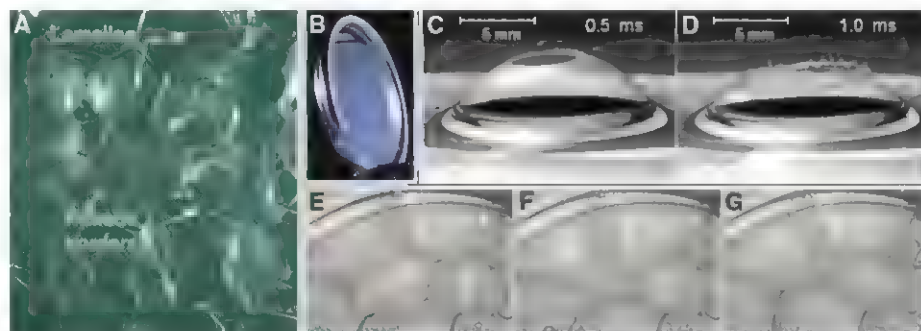


Fig. 1. Physics of foam drainage. (A) A foam of soap bubbles made with common washing detergent. (B) Drainage and thin-film interference. A keyring suspended in soap solution makes a film, which then drains owing to gravity. The subsequent variations in film thickness create interference patterns when lit with white light. (C and D) Rupture of a lamella. [Reproduced from (2) by permission from Macmillan Publishers Ltd, *Nature*, copyright 2010] (E to G) Rearrangement. A lamella [center of (E)] bursts, leading to macroscopic rearrangement of a foam.

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thin, it ruptures and its liquid contents are redistributed, destroying the macroscopic equilibrium of the remaining membranes. Driven by macroscale gas dynamics and surface tension, these other membranes, as well as their film thicknesses, further change as they contort, stretch, and settle into a new equilibrium, setting the stage for continued fluid drainage.

These processes take place over six orders of magnitude in space and time. The liquid in the thin films, though only micrometers thick, drains over tens or hundreds of seconds (Fig. 1B) until a membrane ruptures (Fig. 1, C and D). Membranes burst at hundreds of centimeters per second (2), causing macroscopic rearrangement of bubble topology through surface and fluid forces occurring over less than a second (Fig. 1, E to G). Considerable mathematical analyses, as well as numerical and experimental studies, have focused on these individual components. These include studying the geometry of stable foams—for example, in Plateau's laws (3)—and computational methods to find minimal surfaces (4, 5); evolutionary laws for foam geometry, such as the two-dimensional (2D) von Neumann–Mullins law (6, 7), its extension to three dimensions (8), and statistical variations (9); thin-film equations for drainage in stationary films (10, 11), as well as drainage equations in stationary networks of Plateau borders (12); and experimental studies of topological changes in 2D foams (13). In addition, computa-

tional tools aimed at specific aspects of macroscopic rearrangement include numerical studies of multiphase fluid flow separated by massless and infinitely thin interfaces that do not drain or rupture (14, 15); foam studies based on 2D hydrodynamics (16); and contributions made by the Surface Evolver software (17) in computing minimal energy states of complex configurations.

Here, we exploit the idea of scale separation to introduce a multiscale model that separates foam dynamics into a cycle of three distinct stages, coupling different scales across space and time. These stages are (i) a rearrangement phase, in which a foam out of macroscopic balance undergoes rearrangement due to surface tension and gas dynamics, leading to an equilibrium; (ii) a liquid drainage phase, in which the foam is essentially in macroscopic equilibrium, and the microscopic flow of liquid is modeled until a lamella becomes too thin; and then (iii) a rupture phase, in which a lamella ruptures, sending the foam out of macroscopic balance, after which step (i) is invoked and the process repeated. Together, the dynamics of each phase affects the next, leading to a multiscale model that captures the key effects of foam rearrangement, liquid drainage, and rupture.

Our scale-separated model assumes that the gas and liquid flow are incompressible within the time and space scales under consideration; liquid evaporation in the lamellae occurs during

a longer time scale than rearrangement, drainage, and rupture; and the liquid-gas interface has a no-slip boundary condition with a uniformly constant surface tension. Additional forces, scales, and regimes, beyond those included here, play an important role in foam dynamics, although they can be added to this framework. Diffusive coarsening, which results from gas exchange between bubbles separated by permeable membranes, is important over very long time scales (minutes to hours) (1); although our macroscale Navier-Stokes fluid solver easily allows such permeability effects, our thin-film equations are derived assuming a static equilibrium, and thus we do not include coarsening effects. At the liquid-gas interface, we idealize that Marangoni forces, which act to equilibrate surfactant concentration, occur quickly enough to produce a uniformly constant surface tension, while the no-slip boundary condition assumption ignores some effects of surface rheology, which can be important for surfactant solutions exhibiting mobile boundary conditions. Finally, we focus on dry foams [i.e., foams with liquid occupying less than $\sim 10\%$ of the total volume (1)], though the methodology below has extensions to wet foam modeling as well.

Turning to the individual phases, consider first the rearrangement phase, in which the foam structure is out of macroscopic equilibrium. Surface tension at the liquid-gas interface influences

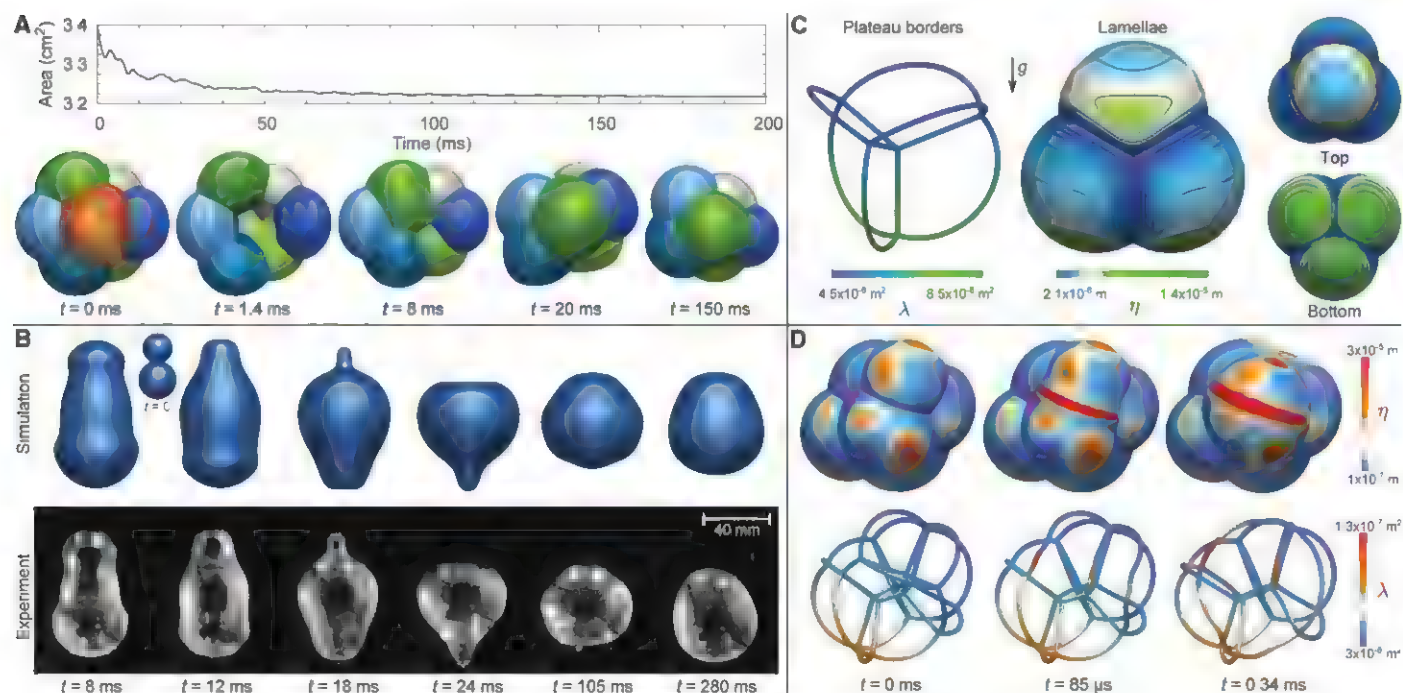


Fig. 2. Verification of numerical methodology. (A) A small cluster, initially in equilibrium, undergoes rearrangement due to the removal of a lamella (orange) at time $t = 0$. Total surface area of the cluster is shown in the plot; see also movie S1. (B) Comparison of numerical results with experiment. Two spherical soap bubbles merge at $t = 0$, subsequently causing surface-tension-driven oscillations that eventually lead to a larger spherical bubble. [Experimental results reproduced from (23) by permission of IOP Publishing]

Numerical simulation uses identical physical parameters and time scale (see table S1). (C) Solution of the coupled lamella and Plateau border thin-film equations on a pyramid of spherical bubbles. Colors indicate thickness η of the lamella and cross-sectional area λ of the Plateau border, and the black lines are contour lines of η . (D) Evolution during rupture. An internal lamella joining the two front facing bubbles ruptures and is removed, leading to rearrangement of bubbles and varying film thicknesses; see movie S2 for an animated view.

the gas dynamics, which in turn evolve the network of lamellae and Plateau borders, rearranging the system of bubbles. Liquid contained in the thin films and Plateau borders is conserved and transported during this readjustment. Because macroscopic fluid mechanics determine the motion, we idealize the membranes as massless and vanishingly thin, thereby approximating their inertial effects as negligible. Mathematically, this leads to the incompressible Navier-Stokes equations for the gas phase, with continuity of the velocity field across the liquid-gas interface Γ , and an effective surface tension of 2σ (i.e., twice the coefficient of a single liquid-gas interface). The interface is thus advected by the velocity field $\mathbf{u}$ of the gas, satisfying

$$\rho_g(\mathbf{u}_t + \mathbf{u} \cdot \nabla \mathbf{u}) = -\nabla p + \mu_g \Delta \mathbf{u} - 2\sigma \kappa \delta_\epsilon(\Gamma)$$

where μ_g is the viscosity of the gas and ρ_g is its density. We have implemented surface tension with a continuum approach (18, 19), whereby the force, existing only at the interface, becomes a body force $2\sigma \kappa \delta_\epsilon(\Gamma)$ through the use of a smoothed Dirac delta function with support concentrated at the interface. The resulting dynamics naturally enforce 120° angle conditions at Plateau borders obeyed by dry foams and allow the interface to change topology (14, 15).

During rearrangement, the liquid in the lamellae and Plateau borders is transported by the motion of the interface in such a way that the amount of liquid is locally conserved. We exploit the thinness of the lamellae and Plateau borders by describing their “thickness” with a scalar function, allowed to vary in space and time. For the lamella, its half-thickness is defined as η , and for the Plateau border, we define λ as the cross-sectional area at any particular location in space; see fig. S1. For liquid contained in the lamellae, conservative transport is modeled by requiring that

$$\frac{d}{dt} \int_{S(t)} \eta = 0$$

where $S(t)$ is any surface patch on $\Gamma(t)$ passively advected by the velocity field $\mathbf{u}$. This model conserves the mass of liquid in the lamellae by measuring the amount of stretching in the interface, and it allows surface currents at the interface to move the liquid tangentially. Liquid in the Plateau borders is conserved with an analogous conservation law.

During the liquid drainage phase, the foam is essentially in macroscopic equilibrium, which means that the dynamics of the gas phase may be taken as negligible, and the surface area has been locally minimized, hence individual lamella have constant mean curvature. We thus require a model for liquid drainage in the (fixed) network of lamellae and Plateau borders. By capitalizing on the inherent scales involved and following the philosophy of “thin-film approximations” (10, 11), which describe the evolving membrane thickness in a single lamella, we build thin-film approximations for drainage in the curved lamellae, as well as the Plateau borders, and devise inter-

related boundary conditions that couple the regions together.

For a lamella of constant mean curvature, we have the partial differential equation (PDE) (see the supplementary online text for further details on the derivation),

$$\eta_t + \frac{1}{3\mu} \nabla_s \cdot (\sigma \eta^3 \nabla_s ((k_1^2 + k_2^2) \eta + \Delta_s \eta) + \rho_g \eta^3) = 0$$

where μ is the viscosity of the liquid, ρ is its density, and $\mathbf{g}_s$ is the component of gravity tangential to the surface. Here, ∇_s is the surface gradient, $\nabla_s \cdot$ is the surface divergence, and Δ_s is the surface Laplacian on the curved surface of the lamella, while k_1 and k_2 are its principal curvatures. This is a fourth-order PDE and needs two boundary conditions on the boundary of the lamella. One condition is chosen to be that of zero Neumann: $\partial \eta / \partial \mathbf{v} = 0$, where $\mathbf{v}$ is tangent to the lamella and orthogonal to its boundary. The other is provided by a flux boundary condition (20, 21) that implements suction of liquid into the Plateau borders at its boundary. The amount of flux is determined by matching the thickness of the lamella to the cross-sectional curvature of the Plateau border under a local Stokes flow argument. In this model, we include effects of surface tension, the curved surface of the lamella, and gravity. Additional physics, such as dis-

joining pressure and van der Waals forces, which are important for films exhibiting long lifetimes, fits into our model by suitably modifying the PDE.

A similar PDE is derived for the thickness of a Plateau border

$$\lambda_t + \frac{C_\Delta}{\mu} \frac{\partial}{\partial l} \left(-\frac{1}{2} \left(\sqrt{3} - \frac{\pi}{2} \right)^{1/2} \sigma \lambda^{1/2} \partial_l \lambda + \lambda^2 \rho_g g_t \right) = S$$

where C_Δ is a constant associated with the cross-sectional shape of the Plateau border, g_t is the tangential component of gravity, and S is a source term representing the incoming liquid from the three lamellae connected to the Plateau border. This equation requires boundary conditions where Plateau borders meet at quadruple junctions, and these are provided by conservation of liquid mass and quasi-static pressure balance.

The rupture phase occurs when a lamella becomes critically thin as a result of drainage. A small tear appears, and the hole in this curved 2D sheet rapidly expands as surface tension causes the membrane to retract. For the bubble sizes considered here, liquid in the membrane retreats to the Plateau borders (2), and this occurs over a time scale that is just a small fraction of the total time it takes for bubbles to rearrange. Although this rupture could itself be treated as an evolving interface within our algorithmic framework, albeit with a more restrictive time-step

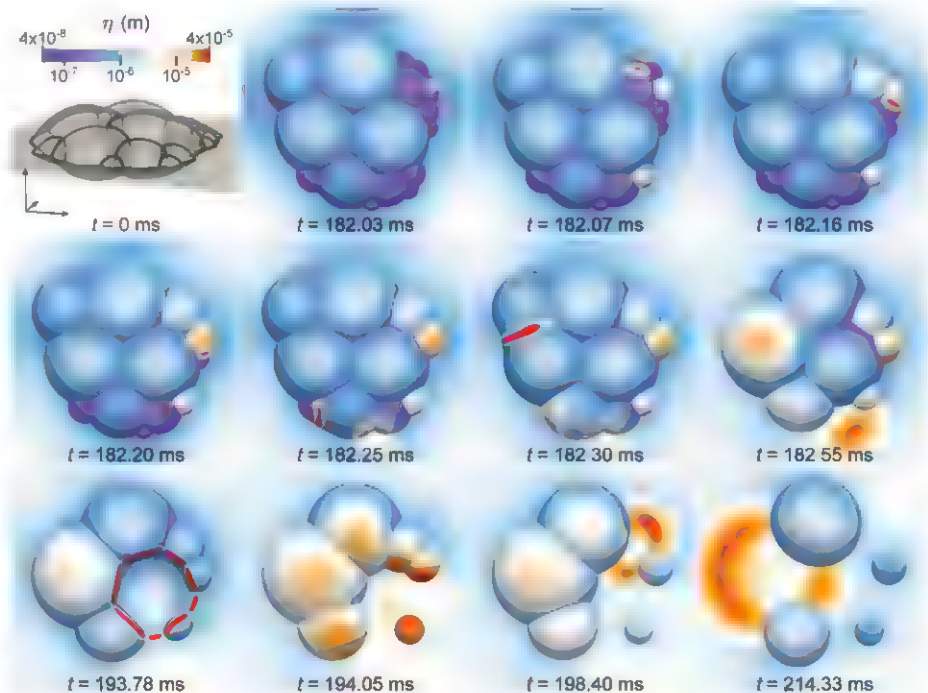


Fig. 3. Results of the coupled multiscale model for a cluster of bubbles attached to a membrane. In the top-left frame, a side view of the initial configuration is shown, using semi-opaque lamellae and emphasizing the Plateau borders, to highlight the 3D structure of the results. In the rest of the frames, a top-down view is given, showing the lamellae film thickness η , corresponding to the indicated color scale. The background membrane absorbs some of the drainage, but is chosen not to rupture. As the system evolves, rupture events can be identified by the localized increases in lamellae thickness. (See movie S3 for the complete simulation.)

requirement in the numerical calculation, we simplify and assume that rupture, once initiated through a prescribed threshold, is instantaneous and that liquid in a ruptured lamella is uniformly distributed to the neighboring Plateau borders.

We now introduce a collection of numerical technologies to accurately compute the solution of these coupled scale-separated equations; more details are given in (19). We solve the incompressible Navier-Stokes equations using a finite difference method implemented on a fixed Eulerian grid, together with a projection method (22) that uses a type of Hodge decomposition to enforce incompressibility when updating the velocity field. The interfaces are tracked with the “Voronoi Implicit Interface Method (VIIM)” (14, 15), which alternates between a finite-difference PDE level set advancement of a single unsigned distance function and a geometric Voronoi reconstruction step, and robustly captures topological change, including transitions at multiple junctions.

To solve the thin-film flow equations, we discretize (i) lamellae as a collection of connected triangulated manifolds, constructed with a new meshing algorithm devised to produce high-quality triangulations, and (ii) the Plateau borders as a network of connected line segments at the junctions of the mesh; see fig. S2. Within each, we design a finite element method to approximate the equations in weak form, using a biharmonic-modified forward time-stepping scheme that ameliorates time-step constraints typically associated with fourth-order nonlinear PDEs. The solutions from the individual lamellae and Plateau borders are coupled together through discretized forms of the flux and quadruple-point boundary conditions. Finally, rupture occurs when the film thickness falls below a chosen minimum value, followed by the macroscopic rearrangement phase.

Before applying our framework to modeling the complete system, we first test and verify individual components, using physical parameters corresponding to a typical gas and typical soap solution; precise values are given in table S1.

We first consider a cluster of bubbles, initially in equilibrium, and then remove a specific lamella (Fig. 2A). After removal, surface tension drives the cluster into a new configuration, undergoing various topological changes in the process. Figure 2A (top) plots the total surface area as a function of time and shows that it reaches a local minimum; Fig. 2A (bottom) (see also movie S1) illustrates how the “hole” made by removing the lamella is filled in, generating capillary waves as it does so, with 120° angle conditions satisfied throughout the process, ultimately leading to an equilibrium where each lamella has constant mean curvature.

To test the accuracy of our Navier-Stokes solver, we compared numerical results with that of an experiment (23), whereby two spherical soap bubbles merge into one bubble, causing surface-tension-driven oscillations (Fig. 2B). Good agreement between the numerical model and experiment is obtained.

Next, we verify that our finite element thin-film equation solvers correctly model liquid drainage (Fig. 2C). A pyramid of four spheres forms a network of six lamellae and 10 Plateau borders. The lamellae are initialized at time $t = 0$ with a uniform thickness of $\eta = 5 \mu\text{m}$ and the Plateau borders with uniform cross-sectional area $\lambda = 0.05 \text{ mm}^2$. Figure 2C shows the thickness after draining for 16.1 s. The effect of gravity is seen with the accumulation of liquid at the bottom of the lamellae and Plateau borders, while the effect of the flux boundary condition can be observed with the reduced thickness of the lamellae at the junctions.

To demonstrate rupture and redistribution of liquid mass, in Fig. 2D, a cluster of bubbles with nonuniform thickness has been draining, and the internal lamella separating the two front-facing bubbles ruptures immediately after time $t = 0$. The liquid originally contained in the lamella, together with the Plateau borders it was once connected to, is locally distributed to the remaining lamellae, as shown by the sudden increase in thickness. The system, driven by macroscopic rearrangement, quickly moves into a new configuration.

We now turn to the complete physical system, and use the multiscale model to predict the evolution of foam cluster dynamics under the combined effects of rearrangement, drainage, and rupture. In the first example, suppose that the lamellae start with a uniform thickness of η_0 . Scaling arguments (see supplementary online text) applied to the lamella thin-film equation together with the flux boundary condition, suggest that the lamellae thicknesses develop a boundary layer, whose width after a fixed amount of time is $\mathcal{O}(\eta_0^{1/2} \lambda_0^{1/4})$, where λ_0 is a typical thickness of the Plateau border. For typical film thicknesses and drainage times, the length predicted by the scaling is on the order of 0.1 mm and was confirmed by numerical tests, as is the result that Plateau borders tend to have the same order of magnitude thickness across the entire network. It follows that for a cluster of bubbles that initially have the same lamellae thickness, all lamellae drain at approximately the same rate, and thus those bubbles smaller than the boundary layer will thin more rapidly and rupture first.

To demonstrate this behavior, and how it effects rearrangement of bubbles, an example is shown in Fig. 3 (see also movie S3 and table S1 for additional details). A cluster of 17 bubbles is suspended by a membrane, so that bubbles protrude below and above the membrane. We designed this configuration in order to make the rearrangement simpler to visualize with a top-down perspective. The cluster has a range of bubble sizes, from 0.1 to 0.5 mm in diameter, and at time $t = 0$ is initially in equilibrium, such that each lamella has a uniform thickness of $10 \mu\text{m}$, and each Plateau border a uniform cross-sectional area of 0.002 mm^2 . After draining for a time of 182 ms, some of the smallest lamellae rupture in quick succession. As this occurs, adjacent bubbles grow in size and increase in thickness. Initially, much of the rupture events are associated with the smaller lamellae, but because rupture affects the macroscopic dynamics of the bubbles, in some cases, larger lamellae rupture as a result of membrane stretching. On this small spatial scale, the rearrangement phase typically takes 0.1 ms to equilibrate, whereas drainage steps takes tens of milliseconds. The results show how a nontrivial sequence of rupture events is obtained, in that bubble rearrangement affects rupture events, both locally and globally, owing to changes in film thickness and macroscopic hydrodynamics.

In another example, we consider a cluster of 27 bubbles, with a larger typical bubble diameter



Fig. 4. Collapse of a foam cluster. Results shown with physically accurate thin-film interference, using a beach scene to provide environment lighting. (See movie S4 for the complete simulation.)

of 3 mm (Fig. 4; see also movie S4). In the figures, thin-film interference is used to show the evolution, by using the lamellae thickness η to solve the Fresnel equations that determine the constructive and destructive interference of reflected light. After draining for 6.4 s, a single bubble bursts, which causes a rapid collapse of the foam structure. Compared to the case in Fig. 3, in this example the typical bubble size is much larger, which makes a priori prediction of rupture events less predictable.

In this work, we have developed a multiscale model of the interplay between gas, liquid, and interface forces for a dry foam, permitting the study of the effects of fluid properties, topology, bubble shape, and distribution on drainage, rupture, and rearrangement. We demonstrated the model by analyzing cascading properties of bubble rupture together with large-scale hydrodynamics. Both the scale-separated model and the underlying numerical algorithms are general enough to allow extension of the physics at individual scales to include other phenomena, such as disjoining pressure, diffusive coarsening, and different types of surface rheology, including liquid-gas interfaces with mobile/stress-free boundary conditions, surface viscosity, evaporation dynamics, and heating. The multiscale modeling

and numerical methodologies presented here suggest a wide variety of related applications, such as in plastic and metal foam formation.

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Supplementary Materials

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Materials and Methods

Supplementary Text

Figs. S1 to S5

Table S1

References (24–27)

Movies S1 to S4

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Spin-Optical Metamaterial Route to Spin-Controlled Photonics

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Spin optics provides a route to control light, whereby the photon helicity (spin angular momentum) degeneracy is removed due to a geometric gradient onto a metasurface. The alliance of spin optics and metamaterials offers the dispersion engineering of a structured matter in a polarization helicity-dependent manner. We show that polarization-controlled optical modes of metamaterials arise where the spatial inversion symmetry is violated. The emerged spin-split dispersion of spontaneous emission originates from the spin-orbit interaction of light, generating a selection rule based on symmetry restrictions in a spin-optical metamaterial. The inversion asymmetric metasurface is obtained via anisotropic optical antenna patterns. This type of metamaterial provides a route for spin-controlled nanophotonic applications based on the design of the metasurface symmetry properties.

Metamaterials are artificial matter structured on a size scale generally smaller than the wavelength of external stimuli that enables a custom-tailored electromagnetic response of the medium and functionalities such as negative refraction (1), imaging without an intrinsic limit to resolution (2), invisibility cloaking (3), and giant chirality (4, 5). An additional twist in this field originates from dispersion-engineered metamaterials (6, 7). A peculiar route to modify the dispersion relation of an anisotropic inhomogeneous metamaterial is the spin-orbit interaction

(SOI) of light; that is, a coupling of the intrinsic angular momentum (photon spin) and the extrinsic momentum ($\delta\mathbf{l}$). Consequently, the optical spin provides an additional degree of freedom in nanophotonics for spin degeneracy removal phenomena such as the spin Hall effect of light (9, 11–14). The chiral behavior originates from a geometric gradient associated with a closed loop traverse upon the Poincaré sphere generating the geometric Pancharatnam-Berry phase (15, 16), not from the intrinsic local chirality of a meta-atom (4, 5, 17). Specifically, spin optics enables the design of a metamaterial with spin-controlled modes, as in the Rashba effect in solids (18–21).

The Rashba effect is a manifestation of the SOI under broken inversion symmetry [i.e., the inversion transformation $\mathbf{r} \rightarrow -\mathbf{r}$ does not pre-

serve the structure ($\mathbf{r}$ is a position vector)], where the electron spin-degenerate parabolic bands split into dispersions with oppositely spin-polarized states. This effect can be illustrated via a relativistic electron in an asymmetric quantum well experiencing an effective magnetic field in its rest frame, induced by a perpendicular potential gradient ∇V , as represented by the spin-polarized momentum offset $\Delta k \propto \pm \nabla V (18\text{--}21)$. In terms of symmetries, the spin degeneracy associated with the spatial inversion symmetry is lifted due to a symmetry-breaking electric field normal to the heterointerface. Similar to the role of a potential gradient in the electronic Rashba effect, the space-variant orientation angle $\phi(x,y)$ of optical nanoantennas induces a spin-split dispersion of $\Delta k = \sigma \nabla \phi$ (22–24), where $\sigma_{\pm} = \pm 1$ is the photon spin corresponding to right and left circularly polarized light, respectively. We report on the design and fabrication of spin-optical metamaterial that gives rise to a spin-controlled dispersion due to the optical Rashba effect. The inversion asymmetry is obtained in artificial kagome structures with anisotropic achiral antenna configurations (Fig. 1, A and B) modeling the uniform ($q = 0$) and staggered ($\sqrt{3} \times \sqrt{3}$) chirality spin-folding modes in the kagome antiferromagnet (25–27). In the geometrically frustrated kagome lattice (KL), the reorder of the local magnetic moments transforms the lattice from an inversion symmetric (IS) to an inversion asymmetric (IAS) structure. Hence, we selected the KL as a platform for investigating the symmetry influence on spin-based manipulation of metamaterial dispersion.

It was previously shown that the localized mode resonance of an anisotropic void antenna

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is observed with a linear polarization excitation parallel to its minor axis (14, 23). We used this anisotropy in artificial kagome structures, where the anisotropic antennas are geometrically arranged in such a way that their principal axes are aligned with the original spin direction in the magnetic KL phases. These metamaterials with the nearest-antenna distance of $L = 6.5 \mu\text{m}$ were realized using standard photolithographic techniques (24) on a SiC substrate supporting resonant collective lattice vibrations [surface phonon polaritons (SPPs)] in the infrared region. We measured angle-resolved thermal emission spectra by a Fourier transform infrared spectrometer at varying polar and fixed azimuthal angles (θ, φ), respectively, while heating the samples to 773 K (see Fig. 1C for the experimental setup). The dispersion relation $\omega(k)$ at $\varphi = 60^\circ$ of the $q = 0$ structure (Fig. 1D) exhibits good agreement with the standard momentum-matching calculation (28) [see (24) for the isotropic KL analysis]. However, the measured dispersion of the $\sqrt{3} \times \sqrt{3}$ configuration (Fig. 1E) reveals new modes as a result of the inversion asymmetry of the structure, which may give rise to an optical spin degeneracy removal. By measuring the S_3 component of the Stokes vector representing the circular polarization portion within the emitted light (24, 29), we observed the $\sqrt{3} \times \sqrt{3}$ spin-projected dispersion and found that the new modes yield a highly

polarized emission with opposite spin states and a Rashba splitting of $\Delta k = 2\pi/3L$ (Fig. 1, F and G).

The removal of the spin degeneracy requires a spin-dependent correction to fulfill the momentum-matching equation. The spin-controlled dispersion of an IaS metamaterial obeys the spin-orbit momentum-matching (SOMM) condition $\mathbf{k}_e^i(\sigma) = \mathbf{k}_{\text{SPP}} + m\mathbf{G}_1 + n\mathbf{G}_2 - \sigma\mathbf{K}_{1,2}$, associated with two differentiated sets of reciprocal vectors: structural and orientational. Here,

$$(\mathbf{G}_1, \mathbf{G}_2) = \frac{\pi}{L}(\mathbf{x} + \mathbf{y}/\sqrt{3}, -\mathbf{x} + \mathbf{y}/\sqrt{3})$$

are the structural reciprocal vectors determined by the $q = 0$ unit cell, whereas $\mathbf{K}_{1,2} = \frac{\pi}{3L}(-\mathbf{x} \mp \sqrt{3}\mathbf{y})$ are the orientational reciprocal vectors determined by a $\sqrt{3} \times \sqrt{3}$ unit cell (Fig. 2A, inset), associated with the local field distribution; $\mathbf{k}_e^i$ is the wave vector of the emitted light in the surface plane; $\mathbf{k}_{\text{SPP}}$ is the SPP wave vector; (m, n) are the indices of the radiative modes; and $i \in \{1, 2\}$ is the index of the specific spin-dependent geometric Rashba term. Note that in the vector equation the sign of the orientational term is determined by the spin. Moreover, the specific geometric Rashba correction is a result of an arbitrary orientational vector choice from $\mathbf{K}_{1,2}$; based on this choice, the secondary vector linearly depends on the primary $\mathbf{K}_i$ and both the structural vectors $\mathbf{G}_{1,2}$. Hence, the SOMM condition arises from the combined contributions of

the structural and orientational lattices resembling the structural and magnetic unit cells in the kagome antiferromagnet; yet, this concept is general and can be tailored to metamaterials and metasurfaces (30). Considering low modes of $(m, n) \in \{0, \pm 1\}$, we calculated the spin-dependent dispersions at $\varphi = 0^\circ$ and 60° (Fig. 2, A and B, respectively), confirming the S_3 measured dispersions (Fig. 1, F and G, respectively), with the optical Rashba spin split of $2\pi/3L$. Such a spin-split dispersion is due to a giant optical Rashba effect, as the geometric Rashba correction is in the order of magnitude of the structural term; in particular, the anomalous geometric phase gradient arising due to the space-variant antenna orientation in the investigated IaS photonic system resembles the potential gradient in the electronic Rashba effect.

The above condition can be also derived from symmetry restrictions, where the representation theory is applied to generate selection rules. If a given structure is invariant under a translation followed by a rotation and both operators commute, then a spin-orbit coupling is expected. By applying the representation theory formalism considering these symmetry constraints, a momentum selection rule with a spin-dependent geometric Rashba correction can be obtained. When this procedure is implemented for the $\sqrt{3} \times \sqrt{3}$ KL, which is invariant under a translation of $2L$ to the left followed by a rotation of 120° counterclockwise, the SOMM condition is realized [see (24) for the detailed discussion].

In addition to the spin-projected dispersions, selection rules can also specify the direction of the surface wave excited at a given frequency (Fig. 2, C to F). Hence, this concept serves as a platform for spin-controlled surface waves possessing excellent potential for manipulation on the nanoscale based on a geometric gradient. Particularly, the SOMM provides the basis for a new type of IaS spin-optical metamaterials, supporting spin-dependent plasmonic launching for nanocircuits (31, 32) and multipoint in on-chip photonics.

The observed optical Rashba spin-split dispersions reveal two obvious relations of (i) $\omega(k, \sigma) \neq \omega(k, -\sigma)$, which is a signature of inversion symmetry violation, and (ii) $\omega(k, \sigma) = \omega(-k, \sigma)$, which is a manifestation of time reversal symmetry (Fig. 1, F and G, and Fig. 2, A and B). Moreover, the spin-projected dispersions show a clear discrimination between the different IaS directions, which is not observed in the degenerated intensity dispersions. By measuring the angle-resolved emission spectra at varying φ and fixed θ , we obtained the strength of the optical Rashba effect pointing on the IS and IaS directions in the KL (24). The S_3 dispersions of the $\sqrt{3} \times \sqrt{3}$ structure are shown in Fig. 3, A and B, where the time reversal symmetry is clearly seen; in addition, the IS directions of $\varphi \in \{30^\circ, 90^\circ, 150^\circ\}$ are manifested by the spin degeneracy, whereas in all other directions the degeneracy is removed.

The symmetry-based approach offers an extended condition because it also recognizes the IS directions resulting in spin-degenerated

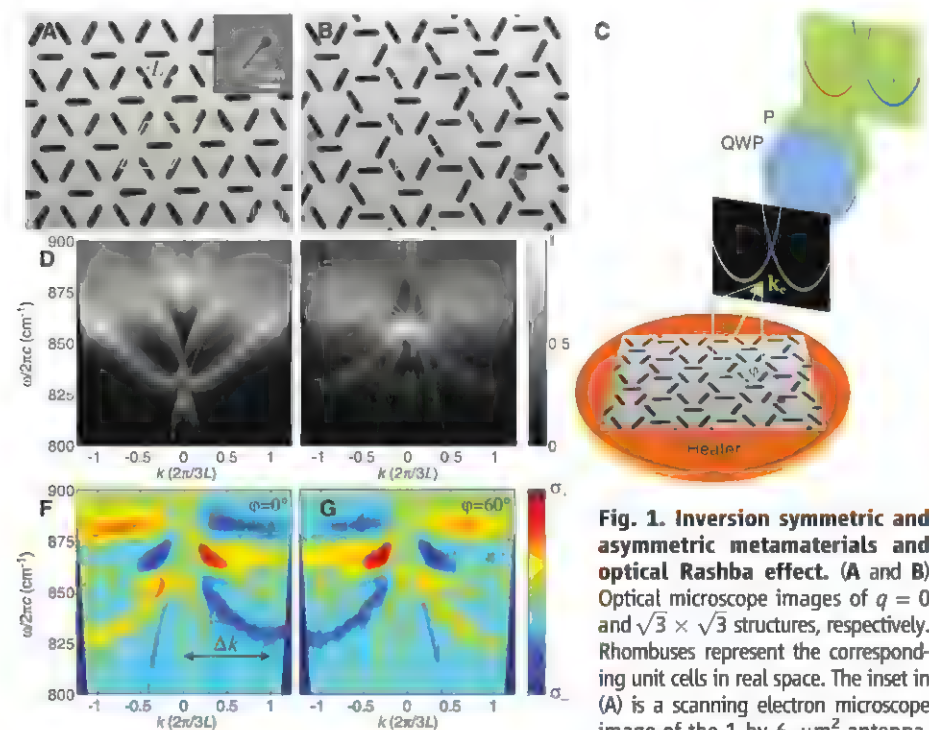


Fig. 1. Inversion symmetric and asymmetric metamaterials and optical Rashba effect. (A and B) Optical microscope images of $q = 0$ and $\sqrt{3} \times \sqrt{3}$ structures, respectively. Rhombuses represent the corresponding unit cells in real space. The inset in (A) is a scanning electron microscope image of the 1-by-6- μm^2 antenna, etched to a depth of 1 μm on a SiC substrate. (C) Schematic setup for the spin-projected dispersion based on the spin-optical metamaterial symmetry. The thermal radiation polarization state is resolved with the use of a circular polarization analyzer [a quarter-wave plate (QWP) followed by a linear polarizer (P)]. $\mathbf{k}_e$, wave vector of the emitted light. (D and E) Measured intensity dispersions of thermal emission from $q = 0$ and $\sqrt{3} \times \sqrt{3}$ structures, respectively. Yellow lines in (D) correspond to the standard momentum-matching calculation; green lines in (E) highlight the new modes. c , speed of light. (F and G) Measured spin-polarized dispersions of the $\sqrt{3} \times \sqrt{3}$ structure along the IaS directions of $\varphi = 0^\circ$ and 60° , respectively.

etched to a depth of 1 μm on a SiC substrate. (C) Schematic setup for the spin-projected dispersion based on the spin-optical metamaterial symmetry. The thermal radiation polarization state is resolved with the use of a circular polarization analyzer [a quarter-wave plate (QWP) followed by a linear polarizer (P)]. $\mathbf{k}_e$, wave vector of the emitted light. (D and E) Measured intensity dispersions of thermal emission from $q = 0$ and $\sqrt{3} \times \sqrt{3}$ structures, respectively. Yellow lines in (D) correspond to the standard momentum-matching calculation; green lines in (E) highlight the new modes. c , speed of light. (F and G) Measured spin-polarized dispersions of the $\sqrt{3} \times \sqrt{3}$ structure along the IaS directions of $\varphi = 0^\circ$ and 60° , respectively.

dispersions. As a reference, we measured the intensity dispersions of the $q = 0$ structure at $\varphi = 30^\circ$, verifying the standard momentum-matching calculation without the geometric Rashba correction. Note that this direction is arbitrary because this structure is IS in all directions; however, it is a specific IS direction in the $\sqrt{3} \times \sqrt{3}$ structure. In accordance with this condition, we did not observe any spin dependence in the measured $\sqrt{3} \times \sqrt{3}$ spin-projected dispersion at this direction. This result is supported by the SOMM condition (Fig.

3D), which is general because it distinguishes between the IS and IaS directions and tailors a spin-degenerated or spin-dependent output, respectively, and it reveals new modes observed in the $\sqrt{3} \times \sqrt{3}$ intensity dispersion (Fig. 3C) owing to the spin-dependent geometric Rashba correction.

The spin degeneracy removal was also shown in the near-field associated with the orbital angular momentum variation along the IaS directions. We revealed a chain of vortices with alternating helicities in the artificial $\sqrt{3} \times \sqrt{3}$

building blocks, carrying a spin-dependent space-variant orbital angular momentum arising from the spiral phase front of the SPPs [see (24) for the detailed analysis]. The reported spin-based phenomena in the near- and far-fields inspire the development of a unified theory to establish a link between the spin-controlled radiative modes and the metasurface symmetry properties to encompass a broader class of metastructures from periodic to quasi-periodic or aperiodic. The design of metamaterial symmetries via geometric gradients provides a route for integrated nanoscale spintronic spin-optical devices based on spin-controlled manipulation of spontaneous emission, absorption, scattering, and surface-wave excitation.

Fig. 2. Spin-orbit momentum-matching and surface-wave control. (A and B) Calculated S_3 dispersions of the $\sqrt{3} \times \sqrt{3}$ structure at $\varphi = 0^\circ$ and 60° , respectively, via the SOMM condition. Red and blue lines correspond to σ_+ and σ_- spin states, respectively. (Inset) Reciprocal space of $q = 0$ (yellow) and $\sqrt{3} \times \sqrt{3}$ (blue) structures with the corresponding reciprocal vectors. The dashed blue arrow indicates that only one of the $\sqrt{3} \times \sqrt{3}$ reciprocal vectors is required to set the dispersion of a spin-optical meta-

material, in addition to both of the $q = 0$ reciprocal vectors. (C and D) Spin-controlled surface-wave concept. The scheme introduces the coupling of the two spin-dependent modes depicted in (A) to surface-wave modes via the degree of freedoms of ω , $k_{in}^{\parallel}$, and σ ; $k_{in}^{\parallel}$ is the component of the incident wave vector k_{in} parallel to the surface. (E and F) Vector summation representation of the SOMM condition forecasting the direction of the surface wave for the aforementioned modes.

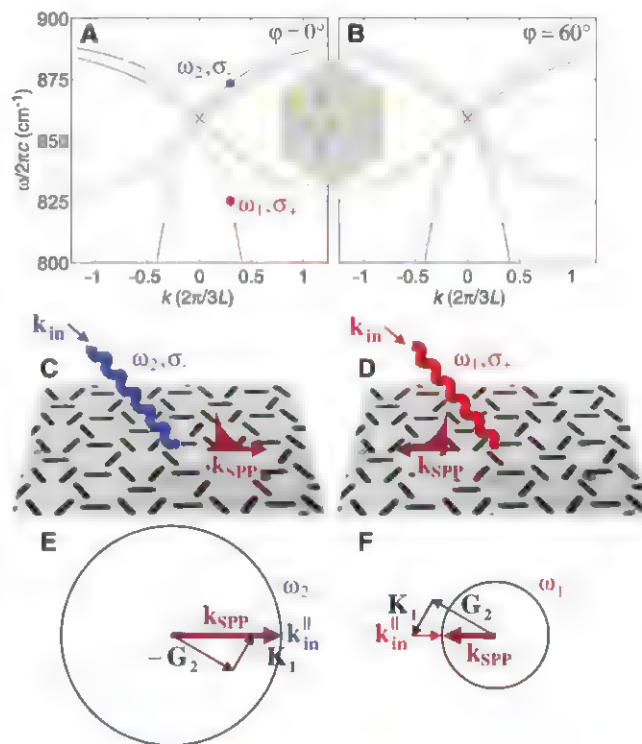
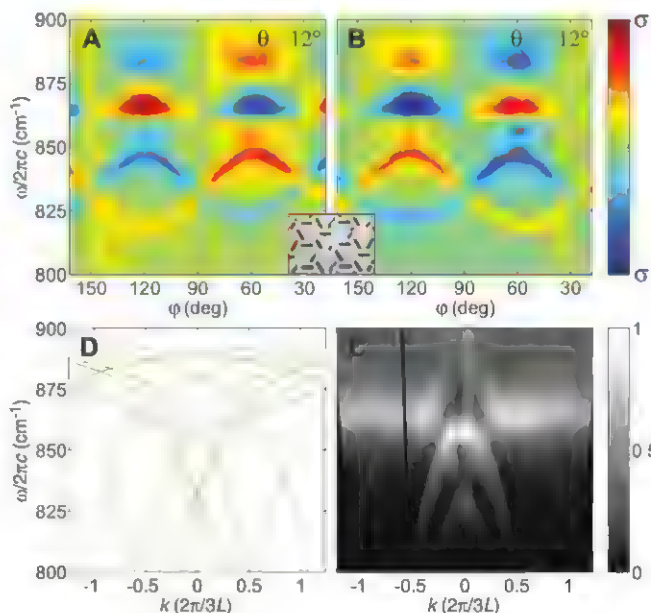


Fig. 3. Symmetry analysis by spin-projected measurements. (A and B) Measured S_3 dispersions of the $\sqrt{3} \times \sqrt{3}$ structure with varying φ at $\theta = 12^\circ$ and -12° , respectively. The inset highlights the IS directions. (C and D) Measured and calculated intensity dispersions of the $\sqrt{3} \times \sqrt{3}$ structure along an IS direction of $\varphi = 30^\circ$, respectively. The standard momentum-matching and the SOMM calculations in (D) are denoted by the yellow and green lines, respectively.



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Supplementary Materials

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Enhanced Role of Transition Metal Ion Catalysis During In-Cloud Oxidation of SO₂

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Global sulfate production plays a key role in aerosol radiative forcing; more than half of this production occurs in clouds. We found that sulfur dioxide oxidation catalyzed by natural transition metal ions is the dominant in-cloud oxidation pathway. The pathway was observed to occur primarily on coarse mineral dust, so the sulfate produced will have a short lifetime and little direct or indirect climatic effect. Taking this into account will lead to large changes in estimates of the magnitude and spatial distribution of aerosol forcing. Therefore, this oxidation pathway—which is currently included in only one of the 12 major global climate models—will have a significant impact on assessments of current and future climate.

The size distribution and properties of atmospheric aerosol particles play a key role in the climate system by affecting how aerosols scatter radiation and modify the brightness and lifetime of clouds (1, 2). The magnitude of aerosol radiative forcing is the largest uncertainty in current climate assessments (3). Processing of particles in clouds changes both their size and hygroscopicity, and is therefore critical in determining the magnitude of aerosol radiative forcing. Sulfate addition to particles, through both in situ oxidation of SO₂ and direct uptake of H₂SO₄ gas and ultrafine particulate by cloud droplets, is the most important in-cloud mass production mechanism (4, 5) (table S1). A detailed understanding of the in-cloud sulfur cycle is therefore critical for accurate estimation of the current and future sulfate distribution and its impact on the climate through aerosol radiative forcing (6). The Hill Cap Cloud Thuringia (HCCT-2010) campaign, a Lagrange-type experiment with measurement sites located upwind and downwind of an orographic cloud as well as within the cloud, enabled investigation of changes in the physicochemical properties of an air mass as it underwent cloud processing. Sulfur isotope abundances were measured in gas-phase SO₂

and particulate sulfate during three cloud events: FCE 7.1, 11.2, and 11.3 (described in text S1 and table S2).

The effect of cloud processing on the particle population and the magnitude of aerosol radiative forcing critically depends on the pathway responsible (e.g., in situ oxidation, H₂SO₄ condensation) for in-cloud sulfate production. Generally, H₂O₂ is consumed early in a cloud's lifetime; thus, most of the sulfate produced from aqueous SO₂ oxidation by H₂O₂ is added to the most cloud condensation nuclei (CCN)-active particles (7), where it has a negligible effect on downwind CCN number concentration and aerosol radiative forcing. Oxidation by O₃ in cloud droplets is very slow at low pH (<5.5) and represents only a small proportion of global in-cloud SO₂ oxidation (8, 9). The climatic effect of sulfate produced from transition metal ion (TMI)-catalyzed oxidation depends on the source of the TMIs: Sulfate produced from oxidation catalyzed by TMIs in fine particles can greatly alter downwind CCN properties and radiative forcing, whereas sulfate produced by TMIs in coarse particles will result in a weaker radiative forcing. Understanding and accurately modeling partitioning between the major oxidation pathways is critical to predict the magnitude and spatial distribution of sulfate aerosol cooling in assessments of future climate.

H₂O₂ is thought to be the major in-cloud SO₂ oxidant (5, 10, 11): Four of the 12 models used in the IPCC Fourth Assessment Report and/or

the AeroCom global intercomparison exercise consider H₂O₂ to be the only aqueous-phase oxidant for SO₂ (table S1). Models including only H₂O₂ and O₃ as in-cloud oxidants attribute 64 to 83% of global sulfate production to aqueous-phase chemistry and 17 to 36% to gas-phase oxidation. They underpredict the observed sulfate concentration by ~20% and overestimate SO₂ concentrations by ~50%, which suggests that a major oxidation pathway is missing or underestimated (12–16). The rate of the TMI-catalyzed oxidation of SO₂ by oxygen is poorly characterized because of the complex interactions among different TMIs (17, 18). $\Delta^{17}\text{O}$ results (19) showed that the contribution of TMI catalysis to sulfate production was strongly underestimated. However, the inclusion of SO₂ oxidation catalyzed by anthropogenic TMIs into the modeled sulfur cycle (GEOS-Chem) has not been able to resolve the discrepancy between modeled and measured SO₂ and sulfate concentrations (20): SO₂ concentrations in the global EMEP domain are overestimated by a factor of 1.8 to 2.7 and sulfate concentrations by a factor of 1.5 to 3.7 for simulations including TMI chemistry; for simulations without TMI chemistry, these same concentrations are overestimated by a factor of 1.9 to 3.0 and by a factor of 0.8, respectively.

Stable sulfur isotopes show distinctive fractionation during chemical reactions and are therefore useful for investigating the in-cloud sulfur cycle. Oxidation by H₂O₂ and O₃ produces sulfate that is enriched in ³⁴S [+15.1 to +19.9 per mil (‰) depending on pH] relative to the reactant SO₂, whereas oxidation by TMI catalysis produces sulfate depleted in ³⁴S (−9.5 ± 3.1‰) relative to SO₂ (21–23). The change in isotopic composition of SO₂ gas between the upwind and downwind stations was used to calculate the fractionation factor for the cloud, α_{cloud} (text S2 and table S3), which was compared with fractionation factors for known reactions to determine the dominant SO₂ oxidation pathway occurring in the cloud (Table 1). The α_{cloud} values clearly show that H₂O₂ or O₃ dominates in-cloud oxidation only during the first nighttime event; during the second nighttime and the daytime event, TMI catalysis is the dominant oxidation pathway. The cloud water pH values were low (fig. S2); thus, oxidation shown isotopically to be due to H₂O₂ or O₃ will be dominated by H₂O₂ and is hereafter referred to as oxidation by H₂O₂. Both the TMI and H₂O₂ concentrations in cloud water at HCCT were within normal ranges during all three

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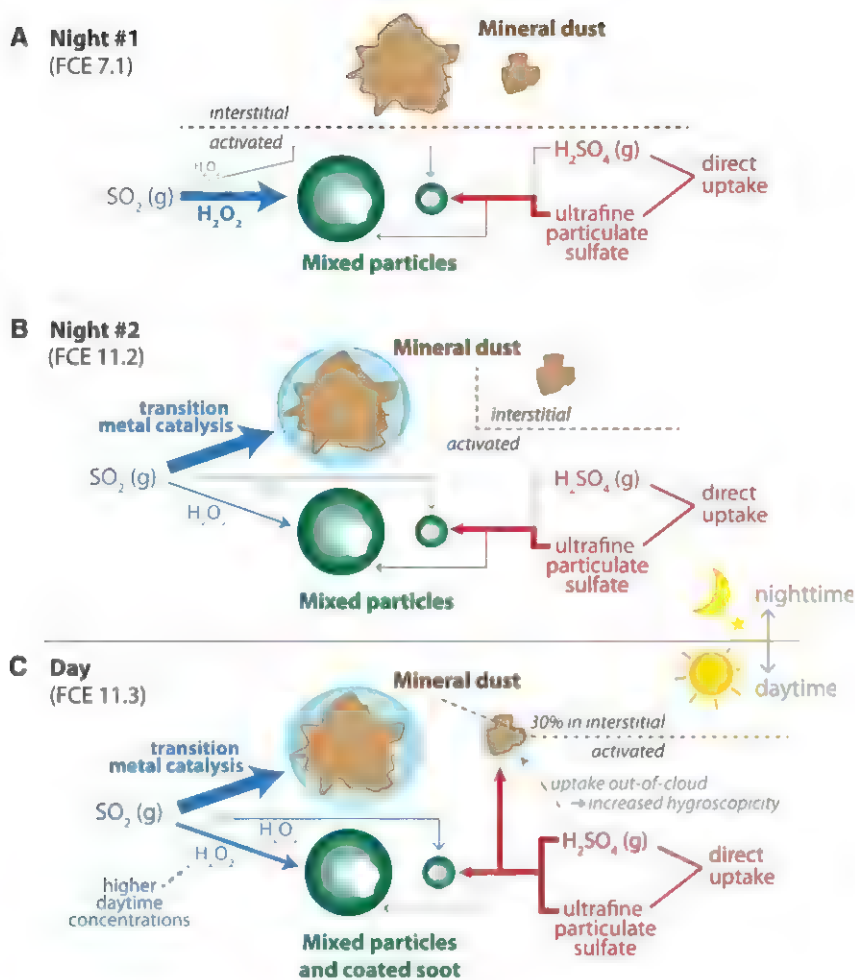
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Table 1. Dominant in-cloud SO₂ oxidation pathway determined from the fractionation of sulfur isotopes. Official event names are shown in brackets. α_{cloud} is the fractionation factor for SO₂ removal in the cloud. Oxidant refers to the oxidation reaction that agrees with the observed in-cloud isotope fractionation; α_{ox} is the corresponding known, laboratory-derived fractionation factor (21).

Event	LWC (g m ^{−3})	α_{cloud} (‰)	Oxidant	α_{ox} (‰)
Night 1 (FCE 7.1)	0.14	11.6 ± 9.8	H ₂ O ₂	15.1 ± 1.3
Night 2 (FCE 11.2)	0.37	−16.1 ± 9.5	TMI catalysis	−9.5 ± 3.1
Day (FCE 11.3)	0.32	−10.8 ± 4.9	TMI catalysis	−9.5 ± 3.1

Fig. 1. Summary of the sulfur cycle occurring in clouds during three measurement periods of the HCCT-2010 campaign. (A and B) The first and second nighttime events, FCE 7.1 and FCE 11.2; **(C)** the daytime event, FCE 11.3. Processes occurring only on particles that activated to form cloud droplets are shown on the “activated” side of the dashed lines. The proportion of activated particles in each class was determined from NanoSIMS (nano-secondary ion mass spectrometry) isotopic mass balance, under the assumption that the droplet residue and interstitial dust remix to form the downwind dust sample (see also table S7). The thickness of the arrow qualitatively represents the relative strength of the flux.



events (7, 24–27) (table S2 and figs. S2 and S3). The dominance of TMI-catalyzed oxidation could therefore be a widespread phenomenon, as suggested previously (19, 20).

Isotopic analysis of single particles, in combination with isotopic measurements of gas-phase sulfur, allowed the major sulfate addition pathways to be resolved for particle class (text S2 and tables S4 and S5). The major sulfate sources to particles are summarized in Fig. 1. Sulfate from H₂O₂ oxidation and direct uptake of H₂SO₄ gas and ultrafine particulate are the two dominant sources of sulfate for activated mixed particles (containing a mixture of secondary organic aerosol (SOA) and secondary inorganic aerosol (SIA); Fig. 2, A and B) and activated fine-mode mineral dust particulate. The oxidation source is more important for larger particles (>600 nm; text S1), whereas direct uptake dominates sulfate addition to smaller particles (<600 nm).

The distinctive fractionation during TMI-catalyzed oxidation showed that it was the dominant sulfate source only for coarse mineral dust (Fig. 2, E and F, >900 nm). Mineral dust represented <1% of the CCN number concentration (table S7); thus, oxidation in cloud droplets nucleated on mineral dust must be extremely fast

to compete with other oxidation pathways and dominate SO₂ removal. Previous results have suggested that anthropogenic TMIs are an important catalyst for SO₂ oxidation in Europe, whereas the low solubility of natural TMIs means that they will be unimportant as catalysts (20). Our results show, however, that natural TMIs catalyzed the dominant oxidation pathway. A recent laboratory study showed that significant amounts of TMIs are leached from natural dust and can rapidly oxidize SO₂ in the aqueous phase (22). The solubility of TMIs in dust may be increased through aging and uptake of acidic compounds such as sulfuric and nitric acid. Anthropogenic TMIs are expected to be concentrated on fine-mode combustion particles, whereas natural TMIs lead to sulfate production in the coarse mode; thus, the radiative forcing potential of the sulfate produced from TMI-catalyzed oxidation by natural TMIs will be much lower than that of sulfate produced by anthropogenic TMIs.

Conditions during the three events were examined to explain why H₂O₂ was the dominant oxidant only during the first nighttime event. The only major difference among the three events is the liquid water content (LWC) (0.14, 0.37, and 0.32 g m⁻³ during the first and second

nighttime events and the daytime event, respectively; see text S2 and table S2). The total concentration of TMIs was similar in all three events; however, differences in the TMI profiles for the three events (fig. S3) suggest that during the first nighttime event, coarse mineral dust was not the major source of TMIs to the cloud water, allowing H₂O₂ oxidation to dominate. Particle size distributions and other results confirm that coarse mineral dust was present in the particle population during the first nighttime event but was unable to activate (text S2 and fig. S4). Our results show that although TMIs are present at HCCT from both anthropogenic and natural sources, the natural mix of TMIs leads to stronger synergistic interactions and faster SO₂ oxidation, and therefore dominates in situ sulfate production.

Comparing the isotopic observations with theoretical calculations for in-cloud SO₂ oxidation during HCCT confirms that the rate of TMI catalysis used in current models results in a strong underestimation of the pathway's importance (text S2). A reaction rate constant for TMI-catalyzed oxidation of S(IV) that considers synergistic effects of different TMIs is available only for the most widely studied system of Fe(III) and Mn(II) (28). Estimations using this rate constant (fig. S5A)

Fig. 2. Characteristic particle types collected during the HCCT-2010 campaign. Images are scanning electron micrographs of filter samples. (A) Accumulation-mode mixed SOA-SIA droplet. (B) Coarse-mode mixed SOA-SIA droplet. (C and D) Soot particles, without (C) and with (D) organic coating. (E and F) Mineral dust particles, without (E) and with (F) organic coating. SOA particles and organic coatings appear dark gray, filter pores appear black, and filter material appears gray. Scale bars, 200 nm [(A), (C), (D)], 1000 nm [(B), (E), (F)].

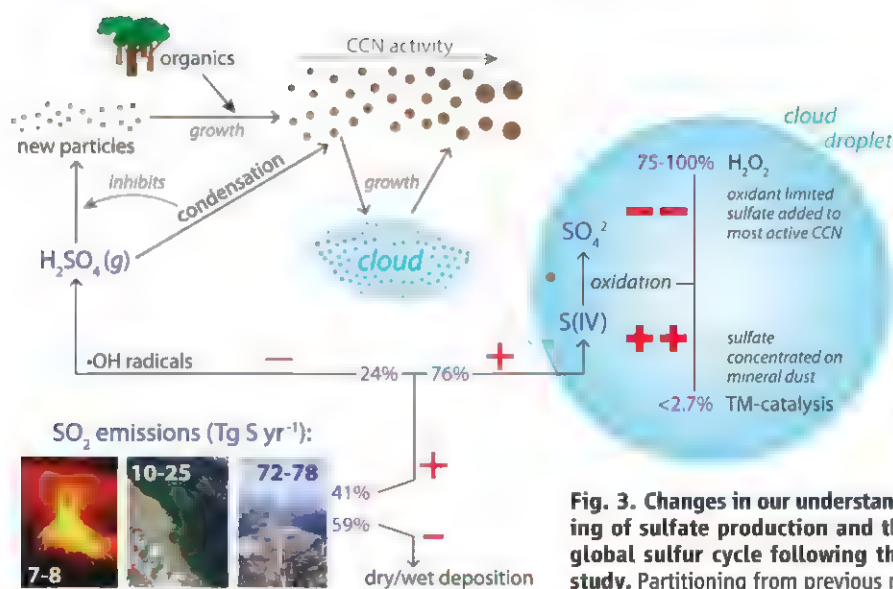
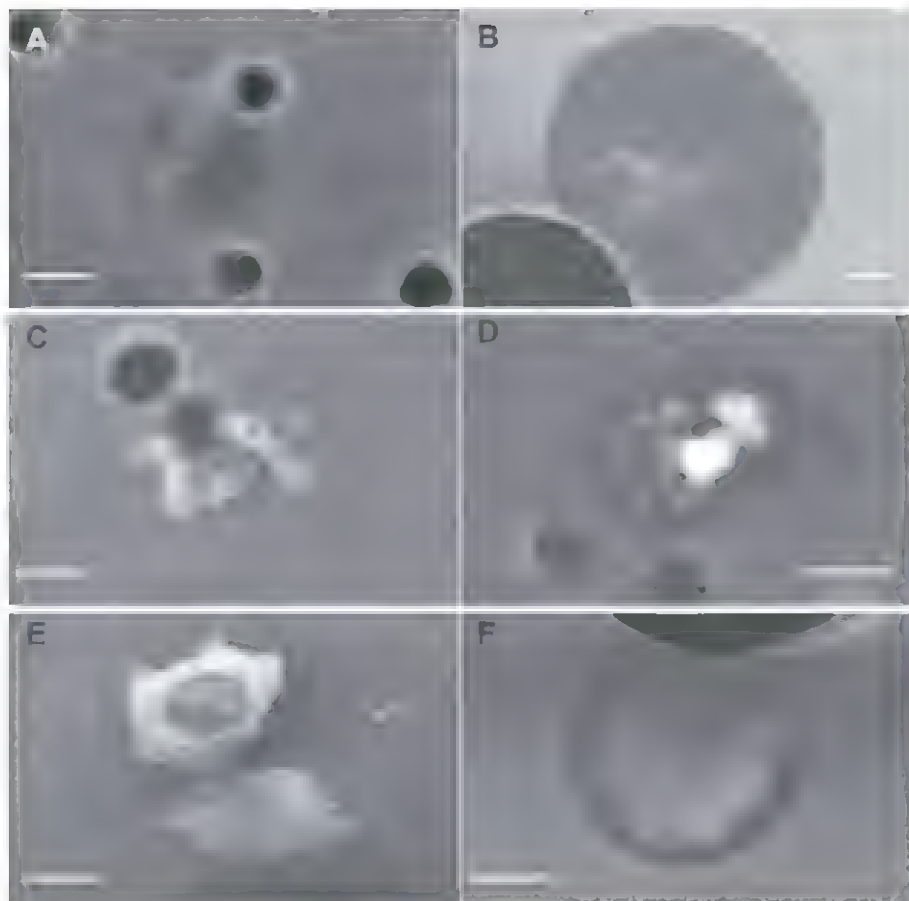


Fig. 3. Changes in our understanding of sulfate production and the global sulfur cycle following this study. Partitioning from previous results (purple) are averages from the

models in table S1. Red plus and minus symbols show how the current study changes our understanding of the sulfur cycle; the number and size of plus and minus symbols represent the expected magnitude of the change and our confidence in it, respectively.

attribute only a few percent of sulfate production to the TMI catalysis pathway. The results of theoretical calculations considering possible interactions of the whole range of measured soluble

TMIs (fig. S5B) show much better agreement with the isotopic measurements.

Because the total TMI concentrations are similar for all three events, the different oxidation

regimes indicated by the S-isotope measurements show that not all metals are equally active in catalyzing oxidation. For example, FCE 7.1 had relatively high Ni^{2+} , Zn^{2+} , and Cu^{2+} concentrations but displayed low TMI-catalyzed oxidation, which suggests that these metals are less active in oxidation, in agreement with laboratory studies (29, 30). Ti is a strong SO_2 oxidant on dust surfaces and could play an important role in catalytic activity. Observations indicate that Ti is leached from natural dust with an efficiency equal to that of Fe (22). The Ti/Fe ratio in upwind particles was higher in FCE 11.2 and 11.3 (average Ti/Fe of 0.05 and 0.03, respectively, versus an average of 0.02 for FCE 7.1). It is possible that the higher Ti/Fe ratio plays a role in the very high reaction rate for TMI-catalyzed oxidation during FCE 11.2 and 11.3.

Reactive uptake coefficients (γ_{obs}) for oxidation of SO_2 by TMI catalysis (text S2 and table S9) can be compared with the sulfur cycle in current models to provide an estimate of how strongly the pathway is underestimated on a global scale. The values of γ_{obs} for mineral dust during FCE 11.2 and 11.3 ($\gamma_{\text{obs}} = 0.03$ to 0.10) are several orders of magnitude higher than the γ value of 10^{-4} used for oxidation of SO_2 on the surface of mineral dust in the ECHAM-HAMMOZ model (31). OsloCTM2 was the only model found that explicitly includes the TMI catalysis pathway

(14); however, the defined maximum modeled rate of $5.56 \times 10^{-6} \text{ s}^{-1}$ is slower than observed during HCCT by one to two orders of magnitude. On the basis of $\Delta^{17}\text{O}$ results, Alexander *et al.* (20) predicted that TMI catalysis contributes 9 to 17% of global sulfate production. Comparison of fractionation factors with previously measured $\delta^{34}\text{S}$ values of SO_2 and sulfate (text S2 and table S10) enables us to predict that the contribution of TMI catalysis to sulfate production varies between 1% (urban) and 58% (rural) at continental sites; it is likely to dominate SO_2 oxidation in all clouds with sufficient supersaturation to activate mineral dust (as summarized in Fig. 3). The results of Alexander *et al.* (20) and Sofen *et al.* (32) suggest that increasing TMI-catalyzed oxidation primarily reduces estimated oxidation by the other aqueous pathways and has little effect on the proportion of SO_2 oxidized in the gas phase, whereas Goto *et al.* (6) found that treatment of aqueous-phase sulfur chemistry in models had the largest impact on sulfate distribution of the parameters tested.

Characterizing the rate of TMI catalysis and including it in models will improve agreement between modeled and observed SO_2 concentrations. The inclusion of the TMI catalysis pathway into GEOS-Chem (20) showed the potential importance of TMI-catalyzed oxidation attributed to anthropogenic TMIs; however, comparisons of models and observations showed a strong overestimation of both SO_2 and sulfate concentrations (12–16). Our observations showed that sulfate produced by TMI catalysis is dominated by natural TMIs and concentrated on coarse mineral dust. It will be removed from the atmosphere relatively quickly, improving agreement between models and observations; it will also result in a minimal modification of the aerosol size distribution and reduced estimates of indirect and direct forcing associated with sulfate from in-cloud oxidation. The estimated level of sulfate aerosol cooling will therefore be much lower and will show a strongly altered spatial distribution, which will have important consequences for assessments of anthropogenic climate change (1). The sulfate produced will also acidify the dust, with important consequences for iron solubility and bioavailability (33). In the coming decades, SO_2 emissions are expected to increase in India and China (34). Windblown dust emissions in both these countries are high; thus, future aerosol cooling may be strongly overpredicted by current climate chemistry models. The results from HCCT provide a size- and particle type-dependent view of in-cloud oxidation that will improve agreement between models and observations and will affect predictions of radiative forcing associated with sulfate in future decades.

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Supplementary Materials

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Networks of bZIP Protein-Protein Interactions Diversified Over a Billion Years of Evolution

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Differences in biomolecular sequence and function underlie dramatic ranges of appearance and behavior among species. We studied the basic region-leucine zipper (bZIP) transcription factors and quantified bZIP dimerization networks for five metazoan and two single-cell species, measuring interactions in vitro for 2891 protein pairs. Metazoans have a higher proportion of heteromeric bZIP interactions and more network complexity than the single-cell species. The metazoan bZIP interactomes have broadly similar structures, but there has been extensive rewiring of connections compared to the last common ancestor, and each species network is highly distinct. Many metazoan bZIP orthologs and paralogs have strikingly different interaction specificities, and some differences arise from minor sequence changes. Our data show that a shifting landscape of biochemical functions related to signaling and gene expression contributes to species diversity.

Differences in transcriptional regulation between species contribute to developmental and functional outcomes (1). Both changes in cis regulatory elements and coding mutations in transcription factors affecting protein-DNA and protein-protein interactions can influence gene regulation (2–5). The basic leucine-zipper

(bZIP) proteins, a large class of multifunctional transcription factors, provide an opportunity to study the evolution of biomolecular interactions. bZIPs can be identified in eukaryotic genomes by a basic DNA binding region followed by a leucine-zipper coiled-coil motif. bZIP proteins can form homodimers and heterodimers via the coiled-coil region, and the dimer that forms influences the DNA sites that can be bound (6). Because bZIP proteins interact with other bZIPs, it is possible to compile a comprehensive list of candidate

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dimerization partners for each protein. Near-complete pair-wise bZIP interactions have been cataloged for human proteins, and many determinants of bZIP dimerization are understood (7, 8). Although bZIP proteins are conserved throughout metazoan evolution, it is unclear whether their dimerization preferences are also conserved.

We measured the bZIP protein-protein interaction networks of five metazoan species that diverged about 1 billion years ago: human (*Homo sapiens*), sea squirt (*Ciona intestinalis*), fruit fly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), and sea anemone (*Nematostella vectensis*). We also investigated two single-cell organisms, a choanoflagellate (*Monosiga brevicollis*), which belongs to the closest sister group of metazoans, and the yeast *Saccharomyces cerevisiae*. We defined 21 bZIP families in humans, containing zero to four paralogs each (9–11) (Fig. 1A). Eighteen families are present in *C. intestinalis*, and 14 can be traced to the last common ancestor of human and sea anemone;

we refer to these as the ancestral bZIP families. A smaller number of these 14 families are recognizable in *M. brevicollis* and *S. cerevisiae* (8 and 4, respectively). Each species also has a number of families that lack clear orthologs in the other six species; we refer to these as novel families (Fig. 1A and table S1).

We quantified interactions between bZIP proteins in vitro using a solution fluorescence resonance energy transfer assay (Fig. 1B) (11). Of 53 human bZIP proteins, 36 were selected to cover the observed sequence diversity (8, 11). For the six remaining species, all pairwise interactions between all identified bZIPs were measured (figs. S1 to S7 and table S2). We confirmed that the data were of high quality and reproducible through repeated measurements of interactions and comparisons with previous studies (figs. S8 to S12 and table S3) (8, 11). Interactions in each species were observed over a range of affinities, and interactions conserved among five metazoans were stronger than those that were partially conserved

or occurred for only one species (Fig. 1C, figs. S1 to S7 and S13A, and table S2). The majority of the proteins in each network (~50 to 90%) were capable of forming homodimers, and only 5 to 30% of all heterodimer combinations tested were observed to interact. However, because the number of possible heterodimers in each network is greater than the number of possible homodimers, the networks of the five metazoan species are composed primarily of heterodimers. For yeast, ~90% of the observed interactions are homodimers. The choanoflagellate network includes an approximately equal number of homodimers and heterodimers (fig. S13B).

We compared the interaction networks of each pair of metazoan species, considering only those proteins with an ortholog in each species. The overlap ranged from 94% of *C. elegans* interactions occurring in human to 33% of human interactions occurring in *D. melanogaster* (table S4) (11). Using the numbers of interactions gained or lost between species to calculate rewiring rates, we estimated

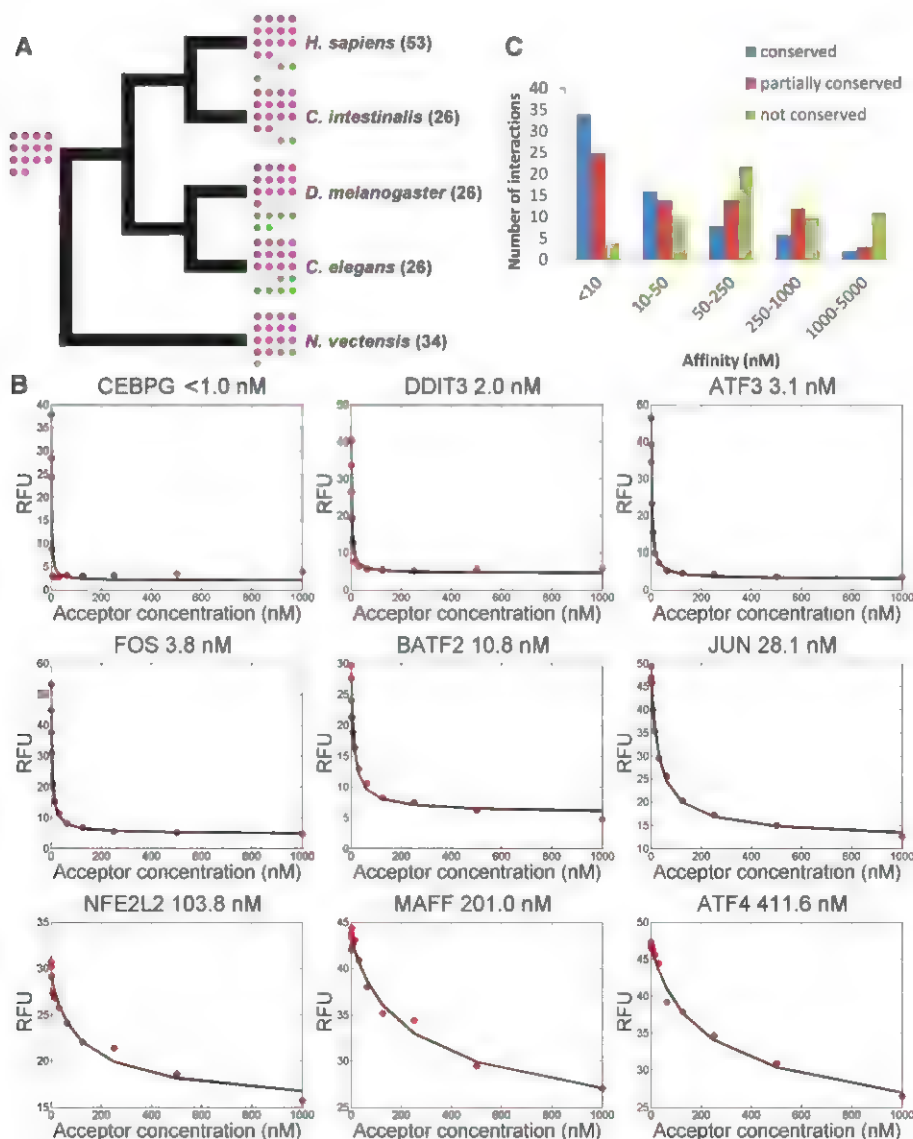


Fig. 1. Quantitation of bZIP protein-protein interactions in five metazoan species. (A) Evolutionary tree for the five metazoan species studied; branch lengths are not to scale. Colored circles represent bZIP families: families in the last common ancestor of metazoans, magenta; families in two or more species, cyan; species-specific families, green. In parentheses is the total number of bZIPs in each organism. **(B)** Binding curves for interactions involving tetramethylrhodamine-labeled ATF4 measured at 21°C, with K_d values indicated. RFU, relative fluorescence units. **(C)** Relationship between conservation and interaction affinity (11).

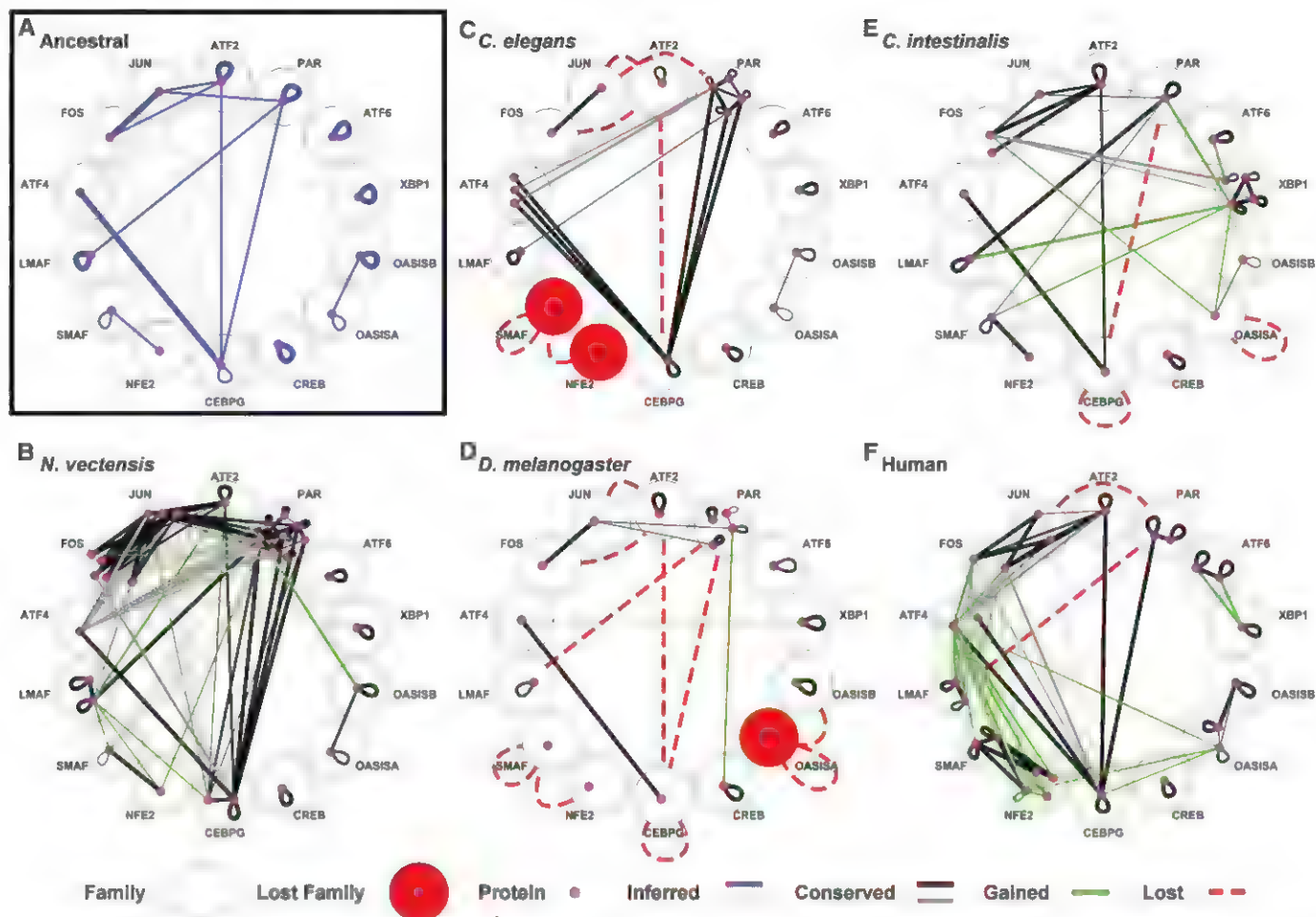


Fig. 2. Changes in interactions between bZIPs in conserved families. Proteins (magenta nodes) are grouped into families (large circles). Edges in the graph represent interactions. In the inferred ancestral network (A), thick dark blue edges show interactions observed in five extant metazoans, narrow dark blue edges show interactions, and light blue edges indicate that interaction in the ancestor is ambiguous (11). In extant species (B to F), green edges show

interactions gained compared with the last common ancestor, red circles highlight lost families, and red dashed lines indicate lost interactions. Black interactions are conserved from the ancestor, and gray interactions may be conserved (status in the last common ancestor is ambiguous). For the extant species, three line thicknesses (widest to narrowest) indicate $K_d < 50$ nM, $50 < K_d < 250$ nM, and $250 < K_d < 1000$ nM. Graphs created using Cytoscape (www.cytoscape.org).

that ~ 0.7 to 2.6×10^{-4} changes per interaction have occurred per million years (table S5) (11). Comparisons to previously reported evolutionary rates are complicated by differences in methodology, but we observed that changes in bZIP interactions occur faster than most estimated protein-protein interaction changes (12, 13). The sequences of bZIP domains have evolved at an average rate compared with other conserved metazoan proteins. Within the domain, the leucine-zipper regions of the bZIPs have evolved more rapidly, and the DNA-binding region is more conserved (fig. S14).

To examine how metazoan interactomes have changed over time, we used parsimony to reconstruct an interaction network for the last common ancestor (11). This network included interactions among proteins in 14 conserved families and contained at least 10 homodimeric and 10 heterodimeric interactions (Fig. 2A); there is ambiguity about additional interactions due to the limited availability of metazoan binding data outside of the five species studied.

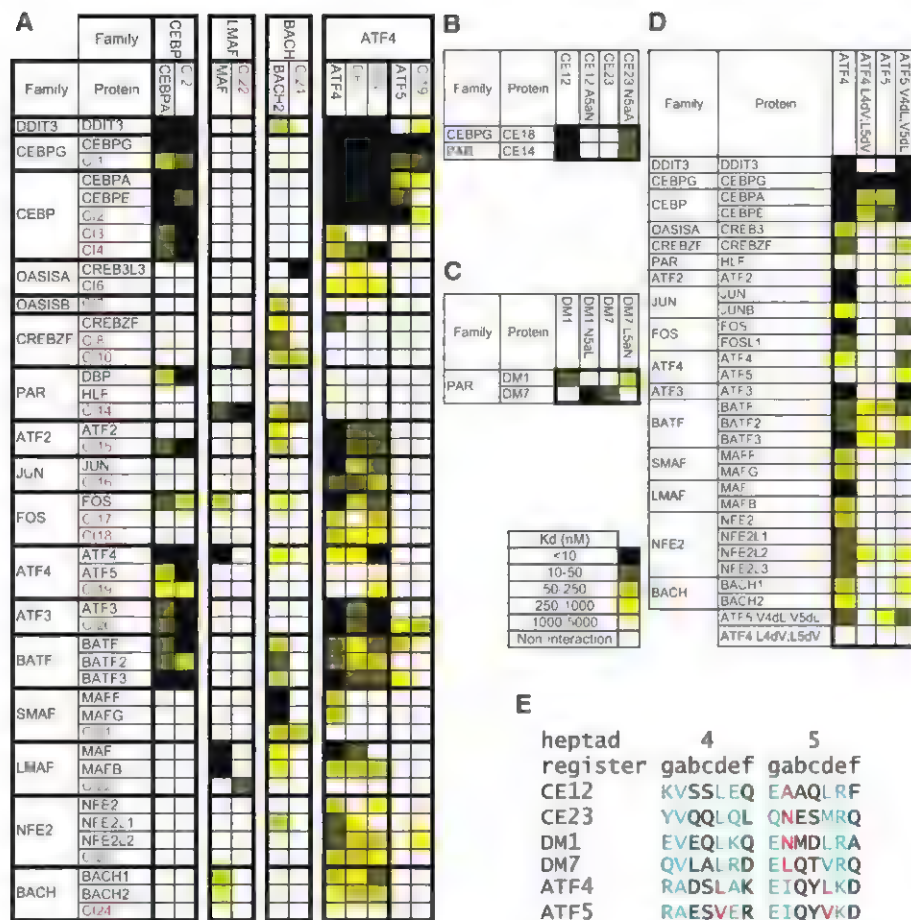
Using an interaction cutoff of $K_d < 1000$ nM, we tracked the gain and loss of interactions among proteins in 14 conserved families (Fig. 2, fig. S15, and table S6). Many interactions were lost in *C. elegans* and *D. melanogaster* (Fig. 2, C and D), whereas many new interactions are observed in *C. intestinalis* and human. In the chordates, new interactions were introduced with C19 (ENSCINP00000010446) in the XBP1 family in *C. intestinalis* and with ATF4 in human (Fig. 2, E and F). Although only a few metazoan proteins have identifiable orthologs in choanoflagellates or yeast, several homodimeric interactions in the inferred ancestral network were observed in these premetazoan species (e.g., ATF6 and ATF2) (figs. S6 and S7).

Each of the metazoans studied here contains bZIPs in families not found in the last common ancestor (Fig. 1A). Four bZIP families originated from gene duplication events involving the ancestral families, e.g., CEBP-CEBPG. Proteins from such pairs often show differences in their interac-

tion profiles (fig. S16). For example, the CEBP protein in flies maintained two of the interactions found in the last common ancestor that were lost by CEBPG (figs. S16 and S17E). Finally, novel families are found in each species that lack orthologs in the other species studied. We observed protein interactions between proteins in novel and conserved families (figs. S1 to S7). Together, rewiring of interactions among ancestral proteins, the addition of conserved duplicated families, and the introduction of novel families (table S6) have allowed each species to evolve a highly distinct bZIP interaction network.

To pinpoint differences in the interaction properties of bZIP orthologs and paralogs by analyzing binding to a common set of proteins, we determined the cross-species interaction network of 56 human and *C. intestinalis* bZIPs. This revealed six families containing members with moderate to highly conserved interaction specificities between human and *C. intestinalis*, e.g., CEBP, and three families with specificities that were less than

Fig. 3. Changes in the interaction specificities of bZIP proteins. (A) Interaction profiles for human and *C. intestinalis* orthologs are in columns, highlighting similar specificities (CEBP), diverged specificities (LMAF and BACH), and the differences among ATF4 family paralogs. Human proteins are black, and *C. intestinalis* red; two *D. rerio* ATF4 paralogs (right column) are green. (B to D) Switching interaction profiles with mutations. Mutants are named by giving the wild-type residue, the coiled-coil heptad number and position as shown in (E), then the mutant residue. (B) Point mutations in *C. elegans* PAR family paralogs CE12 and CE23 switch the interactions of these proteins with CE14 and CE18. (C) Point mutations in *D. melanogaster* proteins DM1 (Pdp1) and DM7 (CG7786) switch the homo versus heterodimerization of these proteins. (D) Two mutations in human ATF4 or ATF5 change the interaction profiles of these proteins to resemble one another. In (A) to (D), binding affinities are presented as heat maps using the scale shown. (E) Sequences of PAR family proteins in *C. elegans* and *D. melanogaster* and ATF4 family proteins in human, highlighting specificity changing mutations. Interface positions are blue, and mutated residues are red.



25% similar between species, e.g., LMAF and BACH (Fig. 3A and fig. S18) (11). Proteins in the ATF4 family have widely varying interaction profiles (Fig. 3A). Human ATF4 has many more interactions than its paralog ATF5. Interactions of *C. intestinalis* ATF4 resemble those of human ATF5, whereas interactions for ATF4 proteins from *Danio rerio* resemble those for human ATF4, indicating that the dramatic expansion in ATF4 versus ATF5 interactions occurred at least ~350 to 400 million years ago (fig. S19).

There is very weak correlation between bZIP sequence identity and interaction specificity (figs. S20 and S21). Therefore, we investigated certain interaction changes in light of known coiled-coil specificity determinants (9). For example, asparagines at coiled-coil a positions are destabilizing when positioned across from hydrophobic amino acids, compared with when they are paired with asparagines (14). *C. elegans* PAR protein CE23 (Zip-7) contains an asparagine at an a position and does not interact with CE14 (Atf-2) or CE18 (Cebp-2), whereas other PAR paralogs do not contain an asparagine at this site and bind these proteins tightly (Fig. 3E). We mutated the asparagine in CE23 to alanine, which is the residue found in PAR protein CE12 (Ces-2), and made the reverse mutation in the CE12 protein. These changes were sufficient to recapitulate the different binding to CE14 and 18 (Fig. 3B). A similar

result was observed for PAR family proteins in *D. melanogaster* on the basis of the same mechanism (Fig. 3C). bZIP interactions can also be destabilized by nonoptimal packing of beta-branched residues (e.g., valines or isoleucines) in the core of the coiled-coil interface (15). Human ATF5 has two consecutive coiled-coil d position valines, which are leucines in ATF4 (Fig. 3E). We mutated the valines to leucines in ATF5 and made the reverse mutations in ATF4. This conferred an ATF4-like interaction profile on ATF5, and the ATF4 mutant also became much more ATF5-like (Fig. 3D). These examples highlight the plasticity of the bZIP interactome, which can be dramatically rewired with changes to just one or two amino acids.

We caution that the interactions observed in vitro in this study do not necessarily occur in vivo. mRNA profiling of human bZIPs shows that most of these proteins are ubiquitously expressed and almost all pairs of bZIPs are co-expressed at measurable levels in at least one tissue (fig. S22) (16), but other factors contribute to whether a bZIP pair will alter gene expression. To begin to investigate the functional consequences of bZIP interaction/noninteraction, we tested DNA binding in vitro for bZIP protein-protein interactions that are not conserved among metazoans (ATF2 with FOS, JUN, and CEBPG and homodimers of PAR and CEBPG). For all families, loss of pro-

tein interaction in a species corresponded to loss of DNA binding (fig. S17). In contrast, four protein interactions conserved in all five species (ATF4-CEBPG, FOS-JUN, and ATF2 and PAR homodimers) were functional for DNA binding in each of the species tested (fig. S17). These observations support changes in bZIP protein interactions as a factor in the evolution of gene regulation.

There is considerable interest in using interactions measured in one species to annotate other organisms (17), but our data suggest a conservative approach to interspecies interaction transfer, at least for large paralogous families. Changes in bZIP protein-protein interactions are common, making them a likely contributor to species diversity. This work provides rich information to guide the study of bZIP homo and heterodimer functions and a resource for investigating the consequences of bZIP network rewiring.

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interaction data are available in the supplementary materials in table S2.

Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6133/730/DC1
Materials and Methods
Figs. S1 to S22
Tables S1 to S6
References (18–48)

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Observing Atomic Collapse Resonances in Artificial Nuclei on Graphene

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Relativistic quantum mechanics predicts that when the charge of a superheavy atomic nucleus surpasses a certain threshold, the resulting strong Coulomb field causes an unusual atomic collapse state; this state exhibits an electron wave function component that falls toward the nucleus, as well as a positron component that escapes to infinity. In graphene, where charge carriers behave as massless relativistic particles, it has been predicted that highly charged impurities should exhibit resonances corresponding to these atomic collapse states. We have observed the formation of such resonances around artificial nuclei (clusters of charged calcium dimers) fabricated on gated graphene devices via atomic manipulation with a scanning tunneling microscope. The energy and spatial dependence of the atomic collapse state measured with scanning tunneling microscopy revealed unexpected behavior when occupied by electrons.

It has long been predicted that relativistic quantum effects can render atoms unstable when the charge of a nucleus Z exceeds a certain critical value Z_c (1, 2). In this supercritical regime, the previously stable atomic bound states are expected to “dive” into the positron continuum and acquire a finite lifetime (3). Quite unlike the standard Bohr state orbitals seen in atomic physics and semiconductor impurity systems, such supercritical quantum eigenstates represent the quantization of a semiclassical trajectory with an electron-like component spiraling inward toward the nucleus (hence the term “atomic collapse”) before spiraling back out and coupling to a positron-like component that propagates away from the nucleus (1, 3–5).

Some efforts to observe atomic collapse states have been made through colliding heavy ions and searching for the positron created when a collapsing electron is torn from the vacuum, but these results remain ambiguous due to the very large

$Z_c \sim 170$ that is required (6, 7). Hopes to realize atomic collapse states rose when theory predicted that highly charged impurities in graphene could display the same atomic collapse physics as atomic nuclei, but with holes in the graphene playing the role of positrons (8–10). Because charge carriers in graphene behave as relativistic particles with a large fine-structure constant value (11–13), the supercritical charge threshold is substantially lower than for atoms, with Z_c on the order of unity (8–10). The atomic collapse state near a Coulomb impurity in graphene should appear abruptly as the impurity charge is increased above Z_c and manifest as a spatially extended electronic resonance whose energy lies just below the Dirac point. Such resonances correspond to the electron-like part of the atomic collapse wave function (in contrast to the far-field measurements of outgoing positrons performed in heavy-ion collision experiments) (8–10). Recently, subcritical Coulomb behavior (i.e., where $Z < Z_c$ and atomic collapse resonances are absent) was observed for charged impurities in graphene (14), but the observation of atomic collapse around supercritical impurities has remained elusive due to the difficulty of producing highly charged impurities.

Here, we report the observation of supercritical Coulomb behavior in atomically fabricated “artificial nuclei” assembled on the surface of a gated graphene device. We synthesized these tunable charge centers by using the tip of a scanning

tunneling microscope (STM) to push together ionized Ca impurities, thus allowing creation of supercritical Coulomb potentials from subcritical charge elements. We used STM spectroscopy to observe the emergence of atomic collapse electronic states extending further than 10 nm from the center of artificial nuclei in the supercritical regime ($Z > Z_c$). By tuning the graphene Fermi level (E_F) via electrostatic gating, we observed behavior in this state that was unanticipated by theory (8–10).

We transferred chemical vapor deposition-grown graphene (15) onto a BN flake placed on a SiO₂/Si surface (the doped Si substrate comprised a gate electrode). The BN substrate reduced the charge inhomogeneity of graphene (16–18) sufficiently for us to observe the intrinsic electronic behavior of pristine graphene adjacent to charged impurities and artificial nuclei. We used Ca dimers as charge building blocks for constructing supercritical artificial nuclei on graphene because they were more easily manipulated with an STM tip than Ca monomers. The dimers formed spontaneously by allowing the monomers to diffuse on the surface by warming to 16 ± 3 K for 1 to 2 min [details of the assembly and characterization are available as supplementary materials (fig. S1) (19)].

To determine the effect of a charged Ca dimer on nearby Dirac fermions in graphene, we measured both the electron- and hole-like local density of states (LDOS) near a single dimer through STM dI/dV (I , current; V , voltage) spectroscopy performed at different lateral distances from the center of a single Ca dimer (Fig. 1A). These spectra exhibited a ~130-meV-wide gaplike feature at E_F caused by phonon-assisted inelastic tunneling, as well as an additional minimum around sample bias $V_S = +0.23$ V associated with the Dirac point (20). These spectra also display a systematic electron-hole asymmetry in that the LDOS intensity of states above the Dirac point increased as the tip neared the dimer, whereas the LDOS of states below the Dirac point correspondingly decreased. This asymmetric behavior is characteristic of subcritical impurities and indicates that Ca dimers were positively charged on graphene at this doping level (14). Such electron-hole asymmetry was observed for Ca dimers at different gate voltages in the range -60 V $\leq V_g \leq +30$ V (fig. S6) (19), and no signature of charge state instability was seen, indicating that Ca dimers were charge-stable within our measurement conditions.

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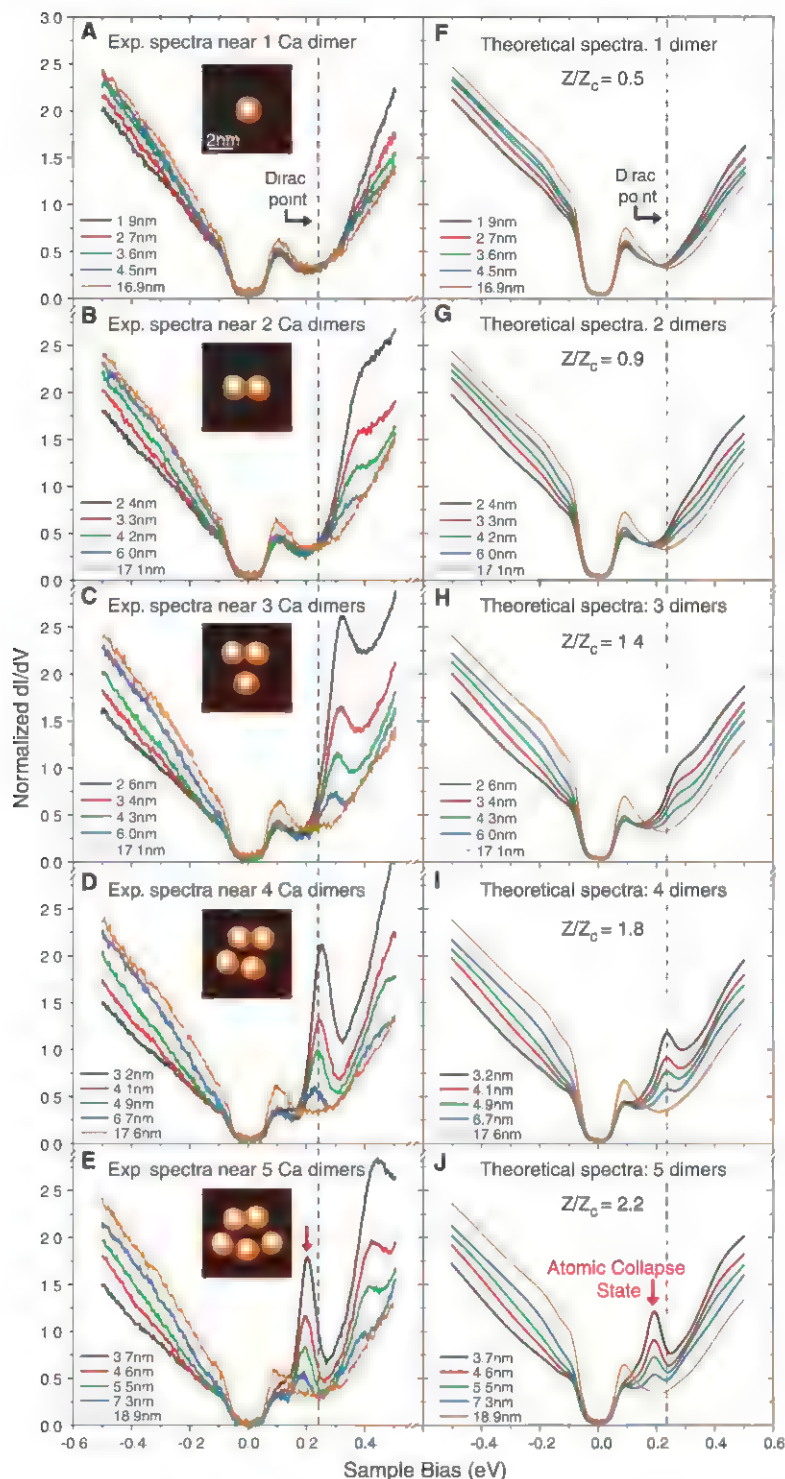


Fig. 1. Evolution of charged impurity clusters from subcritical to supercritical regime. (A to E) dI/dV spectra measured at different distances from the center of Ca dimer clusters (i.e., artificial nuclei) composed of one to five dimers (initial tunneling parameters: $V_S = -0.50$ V, $I = 60$ pA, ac modulation root mean square voltage $V_{rms} = 6$ mV). “Center” here is defined as the average coordinate of dimers within a cluster. All spectra were acquired at the same back-gate voltage ($V_g = -30$ V), and each was normalized by a different constant factor to account for exponential changes in conductivity due to location-dependent changes in tip height (14, 26, 27). (Insets) Scanning tunneling microscopy topographs of atomically fabricated Ca dimer clusters. (F to J) Theoretical normalized dI/dV spectra (obtained from the Dirac equation) for graphene at the same distances from dimer clusters as in (A) to (E) for the nuclear charges Z/Z_c of (F) 0.5 (one dimer), (G) 0.9 (two-dimer cluster), (H) 1.4 (three-dimer cluster), (I) 1.8 (four-dimer cluster), and (J) 2.2 (five-dimer cluster). Black dashed lines indicate Dirac points; red arrows indicate atomic collapse states observed in both experiment and Dirac simulation.

We assembled artificial nuclei containing up to five Ca dimers by using atomic manipulation techniques to push individual dimers into small clusters (see insets to Fig. 1, A to E). dI/dV spectra measured with the STM tip held over the bare graphene near these artificial nuclei revealed the emergence of a resonance in the electronic LDOS (i.e., a quasi-bound state) as the number of dimers in a cluster was increased from one to five (Fig. 1, A to E). Spectra acquired near two-dimer clusters (Fig. 1B) displayed greater electron-hole asymmetry than single-dimer spectra, as well as an extra oscillation in LDOS at high energies above the Dirac point. Spectra taken near three-dimer clusters (Fig. 1C) exhibited even stronger electron-hole asymmetry, and the oscillation in LDOS began to coalesce into a resonance-like structure near $V_S = +0.30$ V. For four-dimer clusters, the intensity of the resonance feature increased markedly and moved down in energy to the Dirac point (Fig. 1D). For the five-dimer cluster, the resonance shifted below the Dirac point (Fig. 1E). The formation of this resonance (or quasi-bound state) as nuclear charge was increased is the “smoking gun” for atomic collapse, as we will shortly describe.

To better characterize the spatial dependence of the quasi-bound state that appears for high- Z artificial nuclei, we performed dI/dV mapping at the resonance energy around a five-dimer cluster (Fig. 2). The resonance intensity displayed a highly symmetric distribution around the five-dimer cluster, despite a marked asymmetry in the arrangement of dimers in the cluster. The intensity of the resonance extended outward from the cluster center to a distance greater than 10 nm. Thus, the resonance was derived from charge carriers moving in the pristine graphene that were spatially separate from the dimers making up the artificial nucleus.

The behavior of the quasi-bound (atomic collapse) state observed for high- Z artificial nuclei depends on whether it is occupied by electrons or empty, as can be seen in the gate-dependent spectra of Fig. 3, which were all measured 3.7 nm from the center of a five-dimer cluster. Red arrows in Fig. 3 indicate the energy of the atomic collapse state at each back-gate voltage, whereas black arrows indicate the energy of the Dirac point extracted by measuring dI/dV spectra on graphene far away (~ 20 nm) from the cluster at each back-gate voltage. In the p-doped regime (-60 V $\leq V_g \leq -15$ V) the quasi-bound state shifted lower in energy (relative to E_F) as more electrons were added to the system, similar to the shift of the Dirac point, except that the resonance transited from above to below the Dirac point as the p-doping was decreased. In the n-doped regime (0 V $< V_g < +30$ V), the resonance shifted below E_F , and its behavior changed dramatically. Here, the resonance intensity decreased and essentially disappeared, although a small rise remained in the dI/dV just above the Dirac point.

The quasi-bound state that develops as the Ca-cluster charge increases is the atomic-collapse eigenstate of a supercritical charged nucleus. This

explanation is supported by Dirac equation–based calculations of the expected LDOS for positively charged nuclei with different charge numbers (8–10, 14), which we compare to our experimental spectra. These dI/dV simulations assume a two-dimensional (2D) continuum Dirac model for undoped graphene in the presence of a Coulomb potential. A boundary condition at a short distance from the nucleus must be chosen to account for the finite size of the clusters. Thus, we set $V(r)$ to the constant value $V(r_0)$ for $r < r_0$ (where r is the distance from the center of a Ca dimer cluster), a similar condition as that used in previous theoretical simulations of supercritical nuclei in a vacuum (1, 2). We fixed the value of r_0 to 1 nm, which matches the geometry of our clusters. We found that varying r_0 over the range $0.5 \text{ nm} \leq r_0 \leq 1.5 \text{ nm}$ did not markedly affect the quality of the fits to our data [simulations using other r_0 values are included in fig. S4 (19)]. The only essential fitting parameter in our simulation was the ratio of the cluster charge to the critical value, Z/Z_c . Here, we define Z as the “effective charge”; that is, the screened cluster charge as seen by Dirac electrons. The value Z absorbs the effects of intrinsic screening due to graphene-band polarization (14) and the substrate. The critical value in this case is $Z_c = \hbar v_F / (2e^2) \sim 0.25$, where $\hbar$ is Planck’s constant h divided by 2π , v_F is the Fermi velocity, and e is the electron charge [this Z_c expression differs slightly from the one used for isolated 3D atoms, see (19) for a detailed discussion]. Additional perturbations from lifetime broadening and electron-phonon coupling are accounted for as in (14) and (21).

Our data suggests that clusters with just one or two Ca dimers are in the subcritical regime, so Z/Z_c here is determined by matching the amplitude of the electron-hole asymmetry between

simulation and experiment according to the method described in (14). Clusters composed of three or more dimers are either transitioning into (three dimers) or have fully entered (four and five dimers) the supercritical regime, as evidenced by the sharpness and energy location of the quasi-bound state resonance (8, 9). For these clusters, Z/Z_c is determined by matching the quasi-bound state resonance energy between the simulation and experiment. The resulting simulated dI/dV spectra from the Dirac equation are plotted in Fig. 1, F to J, next to the corresponding experimental data. The main features seen in our experimental data are reproduced well by the Dirac equation simulations. In particular, our Dirac equation simulations reproduce the increase in electron-hole asymmetry observed in the subcritical regime as the number of dimers increases from one to two, as well as the emergence of the supercritical atomic collapse state as the number of dimers rises from three to five. The Z/Z_c values extracted from our Dirac equation fits are $Z/Z_c = 0.5 \pm 0.1$ (one dimer), 0.9 ± 0.1 (two dimers), 1.4 ± 0.2 (three dimers), 1.8 ± 0.2 (four dimers), and 2.2 ± 0.2 (five dimers) (further details of the simulations, including bare LDOS, can be found in the supplementary materials).

To check that the magnitude of Z/Z_c extracted for Ca dimers from our Dirac equation fits is physically reasonable, we also used *ab initio* density functional theory (DFT) to perform a completely separate calculation of the charge state expected for a Ca dimer adsorbed to graphene. This separate calculation (which had no fitting parameters) yielded a single-dimer charge ratio of $Z/Z_c = 0.6 \pm 0.3$ [only the single-dimer case was calculated using DFT, see (19)]. This finding is in agreement with the value $Z/Z_c = 0.5 \pm 0.1$ obtained via our Dirac equation simulations and, thus, lends

further support to our overall interpretation of the data.

We considered a possible alternative explanation for the quasi-bound state resonance seen for our Ca dimer clusters involving multiple scattering of electrons (22) between different Ca dimers within a cluster. However, this mechanism can be excluded because the distance between Ca dimers is $d \sim 2 \text{ nm}$, which would result in a peak energy measured from the Dirac point of $E = \hbar v_F k \sim \hbar v_F (\pi/d) \sim 1 \text{ eV}$, where k is the momentum of a graphene charge carrier. This value exceeds by more than one order of magnitude the energy of the state observed for Ca clusters on graphene (at most 0.05 eV). The large spatial extent (Fig. 2) of the quasi-bound state resonance shows that it arises from an extended Coulomb field and, thus, also rules out the possibility that it is simply caused by local impurity hybridization with underlying carbon atoms. The observed radially symmetric distribution is consistent with

Fig. 2. Spatial distribution of atomic collapse state around an artificial nucleus. dI/dV map near a five-Ca dimer cluster at the energy of the supercritical quasi-bound state resonance, as marked by red arrow in Fig. 1E (tunneling parameters: $V_s = +0.20 \text{ V}$, $I = 15 \text{ pA}$, $V_g = -30 \text{ V}$). The Ca dimers appear as slightly darker disks near the center of the dI/dV map.

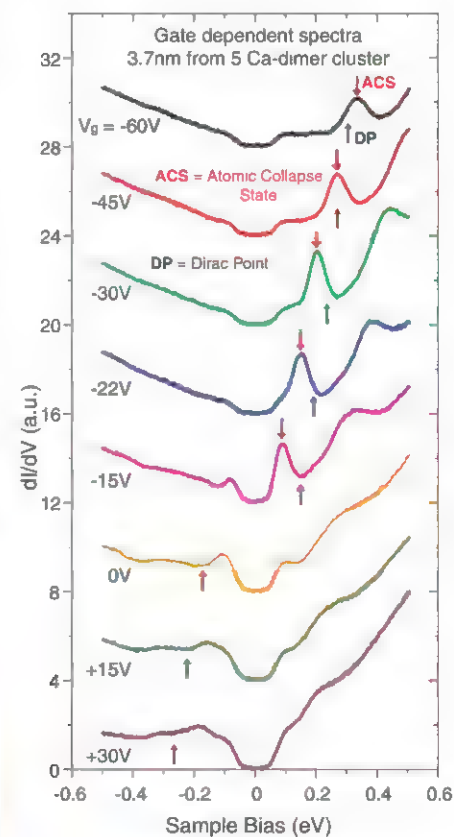
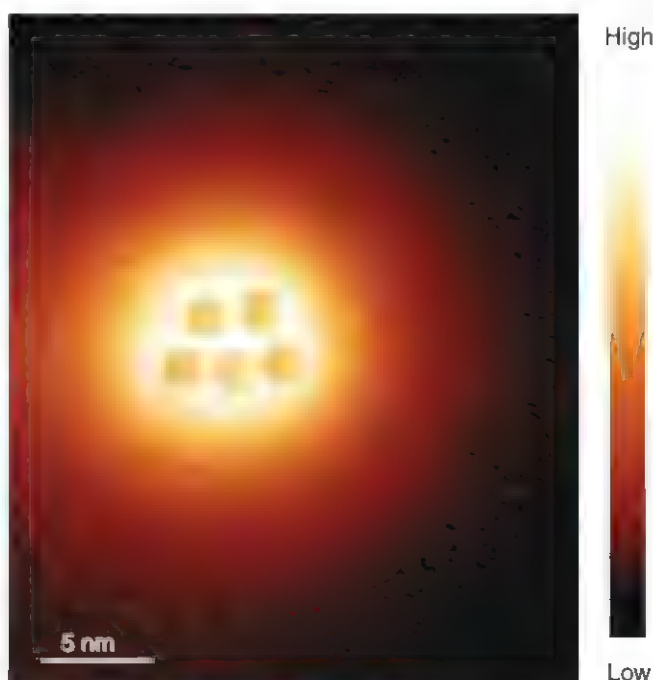


Fig. 3. Dependence of atomic collapse state on doping. Gate-dependent spectra acquired at a lateral distance of 3.7 nm from the center of a five-dimer Ca cluster (initial tunneling parameters: $V_s = -0.50 \text{ V}$, $I = 60 \text{ pA}$, ac modulation $V_{rms} = 6 \text{ mV}$). Curves are shifted vertically for clarity. Red arrows indicate the energy of the atomic collapse state at each back-gate voltage; black arrows indicate the energy of the Dirac point extracted by measuring the dI/dV spectrum on graphene far from the cluster center ($\sim 20 \text{ nm}$) at each back-gate voltage. The atomic collapse state intensity is quenched in the n-doped regime. a.u., arbitrary units.

the predicted behavior of the atomic collapse state having lowest angular momentum ($j = 1/2$) and lowest energy (8, 9). In principle, an infinite number of resonances (corresponding to different principle quantum numbers) should appear in the supercritical regime. However, because their energies follow an exponential sequence and their spatial extent is inversely proportional to their energy measured from the Dirac point (8, 9), only the lowest energy state should be detectable within our experimental resolution (screening suppresses all states with sufficiently large spatial extent).

The strong doping dependence of the intensity of the atomic collapse state (Fig. 3) can be partially explained as a screening effect. In the p-doped regime, the atomic collapse state is experimentally observed to shift to lower energy with respect to the Dirac point for decreased p-doping. This can be explained by free-electron-like screening (23, 24), because reduced screening should result in an increase of the effective charge of the artificial nucleus. The n-doped regime, however, shows a completely different behavior. Here, the amplitude of the atomic collapse state essentially disappears as it moves beneath E_F (i.e., as it becomes occupied with charge carriers). We believe that this behavior arises from internal correlation among the quasi-localized charge carriers that inhabit the atomic collapse state (only one out of the four electron states allowed due to

spin and valley degeneracy can be occupied at any given time) (25). Interaction between these electrons reduces the single-particle spectral function as measured in scanning tunneling spectroscopy, but the precise mechanism for this reduction remains to be determined.

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Robust Circadian Oscillations in Growing Cyanobacteria Require Transcriptional Feedback

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The remarkably stable circadian oscillations of single cyanobacteria enable a population of growing cells to maintain synchrony for weeks. The cyanobacterial pacemaker is a posttranslational regulation (PTR) circuit that generates circadian oscillations in the phosphorylation state of the clock protein KaiC. Layered on top of the PTR is transcriptional-translational feedback regulation (TTR), common to all circadian systems, consisting of a negative feedback loop in which KaiC regulates its own production. We found that the PTR circuit is sufficient to generate oscillations in growing cyanobacteria. However, in the absence of TTR, individual oscillators were less stable and synchrony was not maintained in a population of cells. Experimentally constrained mathematical modeling reproduced sustained oscillations in the PTR circuit alone and demonstrated the importance of TTR for oscillator synchrony.

Circadian clocks are present in all forms of life and are crucial in coordinating physiology with the day and night cycle (1, 2). Clocks can maintain oscillation phase, frequency,

and amplitude for many cycles even in the absence of external cues (3). In the cyanobacterial species *Synechococcus elongatus*, circadian oscillations exhibit remarkable temporal stability with a correlation time of many weeks in constant environmental conditions (4, 5).

Circadian oscillations in *S. elongatus* are generated by a network architecture that consists of two regulatory loops: (i) a posttranslational regulation (PTR) circuit whose output is circadian oscillations in the phosphorylation state of KaiC, an enzyme that catalyzes its own phosphorylation and dephosphorylation in a manner

modulated by the accessory proteins KaiA and KaiB (6–9), and (ii) a transcriptional-translational feedback regulation (TTR) circuit in which the activity of the *kaiBC* promoter is under circadian control (10, 11). The general architecture of coupled PTR and TTR loops is shared by circadian circuits in many species (1, 12, 13).

The role of the TTR and PTR in establishing and stabilizing circadian oscillations has been previously studied both experimentally (9, 14–17) and theoretically (18). The PTR is required for circadian rhythms; abrogation of this circuit results in damped oscillations at the population level (9). However, the persistence of these oscillations is a subject of debate (9, 14). By contrast, the TTR appears to be dispensable under some conditions (15, 16). The PTR circuit alone can generate remarkably stable oscillations in vitro in a reconstituted system (17). Similarly, in vivo circadian oscillations are stable in dark conditions, where transcription is repressed and cells are not growing (6, 19). Theoretical work has suggested that the TTR may be involved in generating robust circadian rhythms during growth (18).

To address the role of the TTR and PTR circuits in vivo in growing cells, we initially constructed two strains (Fig. 1A): (i) a wild-type strain in which both the TTR and PTR circuits are intact, and (ii) a PTR-only strain in which the TTR circuit was abrogated by making expression of the *kaiBC* operon constitutive and at a level similar to the mean expression of *kaiBC* in the wild-type strain (fig. S1). [A strain in which *kaiBC* expression

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is under the control of an inducible promoter was previously characterized but is not a true PTR-only strain; we and others observed residual circadian dependence of *kaiBC* expression in this strain (fig. S1) (11, 20). We monitored the state of the clock by measuring fluorescence derived from expression of a gene encoding yellow fluorescent protein (YFP) under the control of the *kaiBC* promoter (21, 22). YFP intensity reflects the abundance of the phosphorylated form of KaiC that is active in promoting circadian transcription (21).

We measured circadian oscillations over a wide range of growth rates in constant conditions with control of light and composition of the medium by means of a microfluidics device that limits cellular crowding and replenishes spent nutrient buffer (Fig. 1B and fig. S2), permitting long-duration measurements in a chemostatic environment (23). After 2 days of entrainment in a test tube to generate a population of cells oscillating with the same phase, we loaded cells into this device and used automated methods to collect phase contrast (Fig. 1C) and fluorescence images (Fig. 1D) every 20 min for 5 days.

The population-averaged YFP fluorescence (fig. S3), as well as the signal in individual wild-type cell lineages (Fig. 2A), oscillated with circadian periodicity throughout the duration of the measurement. Moreover, a polar plot of the amplitude and phase for each cell lineage confirmed that the initial synchronization of this population, produced by entrainment, persisted throughout the 5-day measurement (Fig. 2B). The PTR-only strain also showed clear circadian oscillations for the population average (fig. S3) and for individual cells (Fig. 2C). After 1 day in constant light conditions, the variation in phase and amplitude within individual PTR-only cell lineages increased (Fig. 2D). Thus, without the TTR circuit, the ability of the PTR-only strain to maintain the phase established by the original entrainment decreases and cell populations lose synchrony.

To quantify the phase stability as a function of time, we calculated the synchronization index (SI) (24). This index is based on the Shannon entropy of the phase distribution (see supplementary text) and varies from one for a completely synchronized population—where all cells oscillate with the same phase—to zero for an unsynchronized population, in which cells oscillate with random phases. The SI for the wild-type strain started high and decreased ~25% over the 5-day measurement (Fig. 2E). The SI for the PTR-only strain started at approximately the same value as the wild-type strain, indicating that disruption of the TTR circuit does not affect entrainment of this strain to light and dark cycles. However, once these external cues were removed, the population of PTR-only cells quickly desynchronized, dropping roughly 70% in SI over 5 days (Fig. 2E). The PTR-only strain

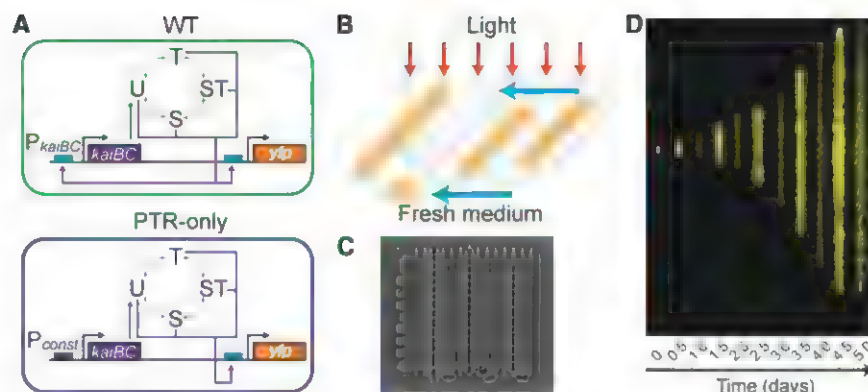


Fig. 1. Long-term measurement of circadian oscillations in *S. elongatus*. (A) Genetic circuit diagrams of the wild-type strain (WT) and the PTR-only strain. KaiC has two phosphorylation sites and transits through four different phosphorylated forms in the PTR circuit during the circadian cycle: unphosphorylated (U-KaiC, represented by 'U'); phosphorylated only on Thr⁴³² (T-KaiC, 'T'); phosphorylated only on Ser⁴³¹ (S-KaiC, 'S'); and phosphorylated on both Ser⁴³¹ and Thr⁴³² (ST-KaiC, 'ST'). In the WT strain, both the KaiC PTR (gray arrows) and TTR (purple arrows) circuits are intact. In the PTR-only strain, the *kaiBC* operon is under the control of a constitutive promoter, abrogating transcriptional regulation. In both strains, a YFP reporter is under the control of the *kaiBC* promoter, whose activity is regulated by the phosphorylation state of KaiC (the PTR). (B) Schematic of growth in the patterned agarose microenvironment. Cells are trapped at the interface between a coverglass and a patterned agarose gel, and a chemostatic environment is maintained by a flow of fresh medium and constant light. (C) *S. elongatus* cells growing in the microfluidics device imaged using phase-contrast microscopy. (D) Fluorescence image collage of subsequent frames of a time-lapse movie of WT cells.

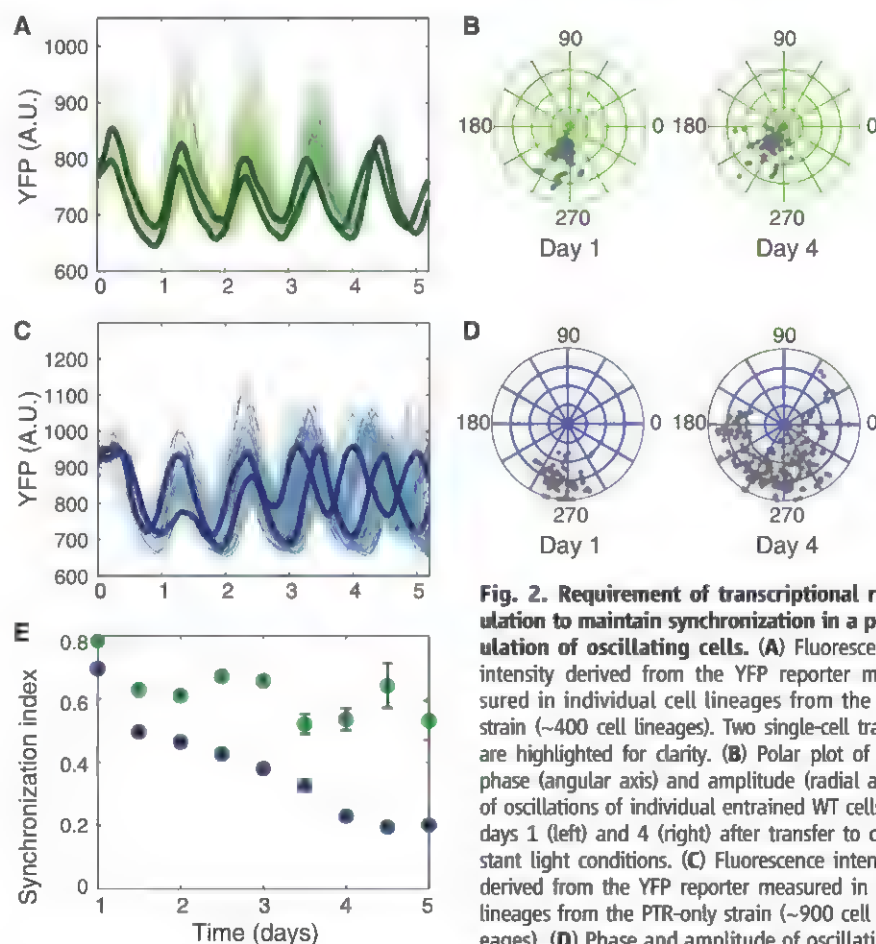


Fig. 2. Requirement of transcriptional regulation to maintain synchronization in a population of oscillating cells. (A) Fluorescence intensity derived from the YFP reporter measured in individual cell lineages from the WT strain (~400 cell lineages). Two single-cell traces are highlighted for clarity. (B) Polar plot of the phase (angular axis) and amplitude (radial axis) of oscillations of individual entrained WT cells at days 1 (left) and 4 (right) after transfer to constant light conditions. (C) Fluorescence intensity derived from the YFP reporter measured in cell lineages from the PTR-only strain (~900 cell lineages). (D) Phase and amplitude of oscillations of entrained cells from the PTR-only strain at days 1 and 4 after transfer to constant light conditions. (E) Synchronization index for the ensemble of cells as a function of time for the WT (green) and PTR-only (blue) strains (see supplementary text for details). Error bars are derived by the bootstrap method.

days 1 and 4 after transfer to constant light conditions. (E) Synchronization index for the ensemble of cells as a function of time for the WT (green) and PTR-only (blue) strains (see supplementary text for details). Error bars are derived by the bootstrap method.

desynchronized more quickly when it was growing faster (one division every 14 to 16 hours; fig. S4H) than the circadian period than when it was growing more slowly (one division every 72 hours; Fig. 2 and fig. S4G). Thus, the TTR had a more important role in stabilizing circadian oscillations for cells with faster growth rates, in qualitative agreement with the predictions of the model we describe below (fig. S5) as well as a previous modeling study (18).

To explore the properties of the TTR circuit, we quantified the dynamics of a mutant *KaiC*^{S431E/T432E} strain lacking the PTR but containing an intact TTR circuit (the “TTR-only strain,” in which the potential for feedback reg-

ulation of *kaiBC* expression exists) (Fig. 3A and fig. S6) (9, 14). This strain showed an initial decrease in fluorescence intensity after transfer to constant light conditions that then increased and stabilized on the first day to an average expression level around which individual cells fluctuated (Fig. 3B). Similar behavior was observed for faster growth rate (fig. S7). The initial dip appeared to be set by the entrainment protocol (fig. S6D) and was not affected by the transfer of cells to the microfluidics device.

To determine which strains exhibit circadian oscillations, we calculated the average of the power spectrum of the fluorescence intensity of individual cell lineages (Fig. 3C). The wild-type

and PTR-only strains had nearly identical power spectra with a prominent peak at the expected 1-day period. A comparable peak was not present for the TTR-only strain, confirming that there were no circadian oscillations in this strain. This result is consistent with population studies that reported damped oscillations of a clock transcriptional reporter in the *KaiC*^{S431E/T432E} strain (9). Thus, the role of the PTR circuit is to establish the oscillations and the role of the TTR circuit is to stabilize these oscillations in growing cells.

To explore the interplay of the PTR and TTR circuits, we created a mathematical model of *in vivo* circadian oscillations in *S. elongatus* that consisted of a PTR pacemaker and a TTR controller (Fig. 3D). The PTR portion of the model was based on a model of the *in vitro* oscillator (7) with addition of terms to account for loss of protein due to degradation and dilution (25). The TTR portion of the model consists of a simple negative feedback loop in which the clock protein *KaiC* represses its own mRNA synthesis. We performed least-squares fitting to our experimental data from the TTR-only strain to derive model parameters that constrain the negative feedback describing the TTR circuit and found that the experimentally observed dynamics of the reporter in the TTR-only strain could be accurately represented by this simple model (Fig. 3E). This model constrained by parameters determined from experiments (table S1) also reproduced the sustained oscillations observed in the population average data for the wild-type and PTR-only strains (Fig. 4A and fig. S3).

To determine whether our model could recapitulate circadian oscillations of the wild-type versus PTR-only strains in individual cell lineages, we performed stochastic simulations of the model using parameters determined from experiments (table S1) (26). We found that the wild-type model produces circadian oscillations (Fig. 4B) that desynchronize only weakly with time (Fig. 4C), although not as weakly as observed experimentally, presumably because of simplifications in the model (such as treating *KaiC* as a monomer rather than a hexamer). The PTR-only model, however, produces circadian oscillations (Fig. 4D) that desynchronize far more rapidly than the wild-type model and that desynchronize more quickly with increased growth rate (Fig. 4, E and F, and fig. S5), as observed experimentally. Repression of *kaiBC* transcription by U-*KaiC* and S-*KaiC* (see legend to Fig. 1 for details of the PTR circuit) creates a peak of *kaiBC* mRNA prior to the peak of U-*KaiC* in the PTR circuit. As a result, newly synthesized U-*KaiC* accumulates coincidentally with the rise of U-*KaiC* from the PTR cycle, and this synchronization of new and existing U-*KaiC* enhances the stability of the PTR cycle, increasing the robustness of the oscillator (18).

To characterize the extent to which the TTR circuit enhances robustness of the circadian oscillator to variability in clock components and

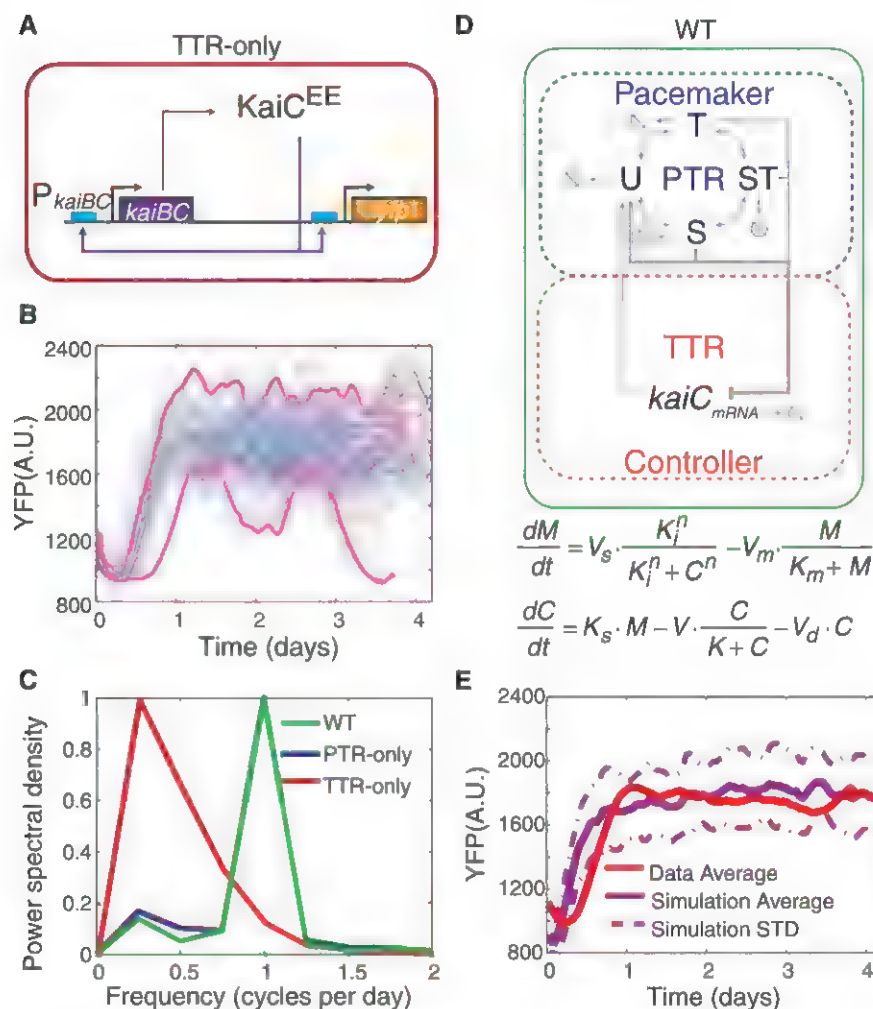


Fig. 3. Insufficiency of transcriptional feedback in the absence of a PTR circuit to generate circadian oscillations. (A) Genetic circuit diagram of the TTR-only strain. (B) Fluorescence intensity derived from the YFP reporter measured in individual entrained cell lineages of the TTR-only strain (~400 cell lineages). Two single-cell traces are randomly selected and highlighted in pink. Cells are dividing once every 24 hours. (C) The average of power spectra of single-cell fluorescence traces from the WT strain (green), PTR-only strain (blue), and TTR-only strain (red). (D) Diagram of the model of the WT circuit. Oscillations are generated by the PTR circuit (pacemaker in the blue dashed box), and abundance of newly made *KaiC* is controlled by the TTR circuit (controller in the red dashed box). “ $\emptyset$ ” represents degradation. Two equations describe the controller dynamics as negative feedback, in which *KaiC* (C) regulates its own mRNA (M) synthesis in a Hill repression function (see supplementary text for details). (E) The average YFP expression (red solid line) for the ensemble of cells from the TTR-only strain. The purple solid line shows the average of the stochastic simulated traces generated from the equations in (D); the purple dashed lines indicate SD of the simulated traces.

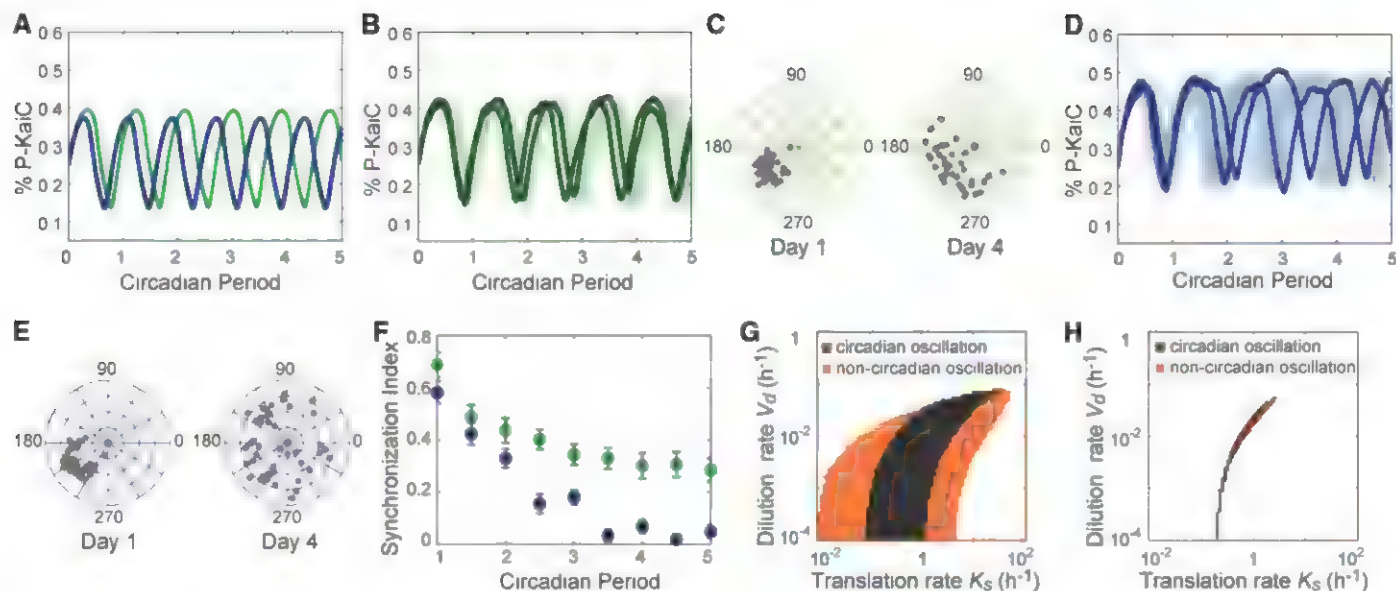


Fig. 4. Requirement of transcriptional feedback for robustness of in vivo oscillations. (A) Deterministic simulations of circadian oscillation of KaiC phosphorylation for the WT (green) and PTR-only (blue) circuits. The simulated period of the PTR-only strain is 6% shorter than that of the WT strain. (B) Stochastic simulated traces for the WT strain. Two of the 100 displayed traces were randomly selected and highlighted. (C) Phase versus amplitude plot of simulated traces for the WT strain at days 1 and 4. (D) Stochastic simulated traces (100 traces) of circadian oscillation of KaiC phosphorylation for the PTR-only strain. (E) Phase versus amplitude plots for simulations of the PTR-only strain. (F) Synchronization index for

the stochastic simulated traces as a function of time for the WT (green) and PTR-only (blue) strains. (G and H) Sensitivity analysis of the oscillation period. Models of the WT (G) and PTR-only (H) systems were numerically solved for dilution and translation rates simultaneously varied over four orders of magnitude. Solutions with periods ± 5 hours around the circadian period of the model output were classified as circadian (black), and other solutions yielding stable oscillations outside this range were denoted noncircadian (orange). We selected ± 5 hours because the same relative range of periods is observed in the measured WT system power spectrum in Fig. 3C.

cellular growth, we performed a systematic sensitivity analysis of the wild-type and PTR-only models. We varied the values of parameters common to both the wild-type and PTR-only models, numerically integrated the wild-type and PTR-only model differential equations, and analyzed the power spectra of the resulting time trajectories to detect circadian oscillations. We find that the volume of parameter space that supports circadian oscillations in the wild-type system (Fig. 4G) is far larger than that of the PTR-only model (Fig. 4H). Furthermore, within the space of parameters supporting circadian oscillations, the wild-type system experiences much smaller changes in period upon parameter variation than does the PTR-only system (Fig. 4, G and H, and fig. S8) (see supplementary text). Thus, the TTR circuit is able to buffer the circadian oscillator against stochastic fluctuations in clock components, and also against sustained changes in parameters such as the cellular growth rate and KaiC translation and degradation rates, all of which cause profound changes in the PTR-only system when varied (fig. S8).

Our findings show that the PTR can generate oscillations in vivo in the absence of TTR feedback. However, without TTR feedback, populations of cells lose synchrony because of phase drift of individual oscillators. Coupling of the PTR pacemaker to the TTR controller generates robustness—insensitivity of period and phase to changes in parameters—that enables the clock to

maintain accurate oscillations in the absence of external cues for long durations and that explains the remarkable stability of the cyanobacterial clock. This architecture may represent a general solution used in other circadian circuits.

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Supplementary Materials

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Materials and Methods
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Figs. S1 to S10
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Global Leaf Trait Relationships: Mass, Area, and the Leaf Economics Spectrum

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The leaf economics spectrum (LES) describes multivariate correlations that constrain leaf traits of plant species primarily to a single axis of variation if data are normalized by leaf mass. We show that these traits are approximately distributed proportional to leaf area instead of mass, as expected for a light- and carbon dioxide-collecting organ. Much of the structure in the mass-normalized LES results from normalizing area-proportional traits by mass. Mass normalization induces strong correlations among area-proportional traits because of large variation among species in leaf mass per area (LMA). The high LMA variance likely reflects its functional relationship with leaf life span. A LES that is independent of mass- or area-normalization and LMA reveals physiological relationships that are inconsistent with those in global vegetation models designed to address climate change.

Leaf size varies by several orders of magnitude between species, and leaf traits are typically normalized by mass or area to study relationships among them. The leaf economics spectrum (LES) (1, 2) has received con-

siderable attention in part because the tight relationships among mass-normalized leaf traits appear to constrain the biodiversity of leaves to a single axis (Fig. 1). The apparent simplicity of this relationship is appealing because of the need

to summarize functionally important aspects of biodiversity in models of the terrestrial carbon cycle and climate change (3–6).

The GLOPNET (Global Plant Trait Network) leaf traits data set (2), from which the LES was generated, reports values of maximum rate of net photosynthesis ($A_{\max}$), dark respiration rate (R_{dark}), nitrogen (N) and phosphorus (P), leaf life span (LL), and leaf mass per area (LMA, which increases with leaf thickness and tissue density) (throughout this paper, we use “N” and “P” to refer to concentrations per unit leaf area or mass, and we use the words “nitrogen” and “phosphorus” to refer generically to the elements). Correlations among traits in GLOPNET [primarily reflecting interspecific variation (7)] are much

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Fig. 1. Observed mass-normalized LES (left three columns) and mass-normalized predictions from an area-proportional null model with no functional correlations involving $A_{\max}$, R_{dark} , N, or P (right three columns). The observed mass-normalized LES and the null model (which leaves intact the actual LMA-LL relationship) have similar overall structure but differ quantitatively due to relationships not caused by mass normalization. All values are $\log_{10}$ -transformed with means rescaled to (0,0).

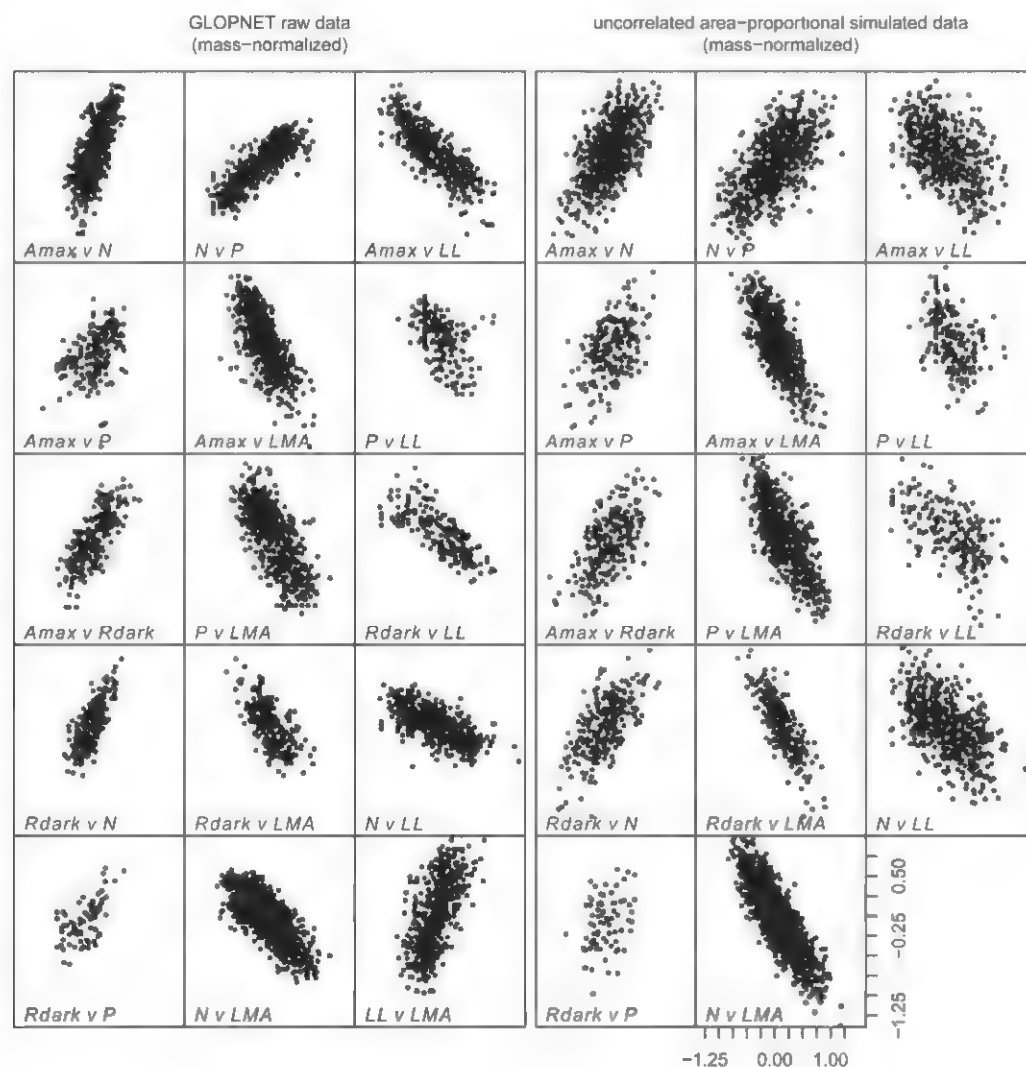
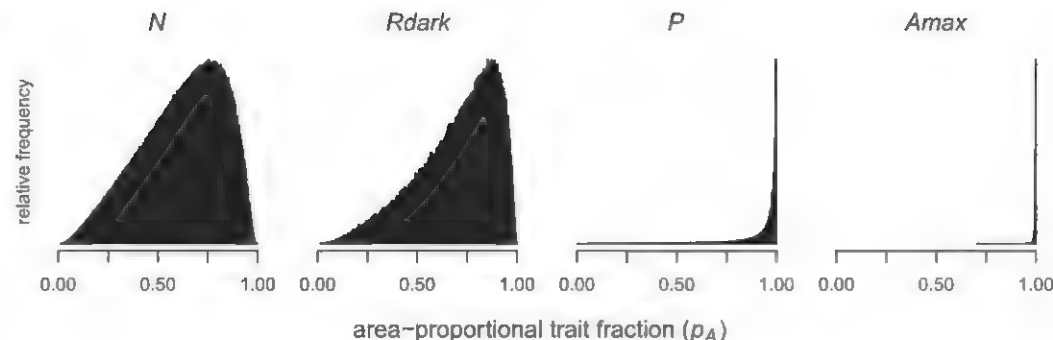


Fig. 2. Frequency of species with area-proportional trait fraction p_A and mass-proportional fraction $1 - p_A$ for N , R_{dark} , P and A_{max} in GLOPNET. A p_A value of 1 indicates pure area proportionality, whereas 0 indicates pure mass proportionality. The histograms are estimated from the posterior distribution of parameters from Model-C and account for parameter uncertainty and the distribution of LMA values in GLOPNET (7). All four traits (especially A_{max} and P) are primarily area proportional for most species.



stronger if A_{max} , R_{dark} , N , and P are normalized by mass than if normalized by area (Fig. 1 and fig. S1).

By definition, a trait is distributed proportional to mass if normalizing by mass makes the trait statistically independent of mass, area, and LMA (7). Similarly, area normalization removes all effects of leaf size from an area-proportional trait. If a trait is mass-proportional, then the total (non-normalized) trait amount in leaves of species with the same leaf mass does not increase with leaf area (i.e., the trait does not vary with LMA among species that share the same leaf mass), whereas if a trait is area-proportional, it does not increase with leaf mass among species with the same area. We statistically partitioned A_{max} , R_{dark} , N and P in GLOPNET into area- and mass-proportional components (Fig. 2) (7). Almost all A_{max} and P are proportional to area in nearly all species, and most leaf N and R_{dark} are area-proportional in more than 90% of species (Fig. 2) (7). If mass- and area-proportionality hypotheses are given equal prior probabilities, then the posterior probability of the former is less than 10^{-25} for each of the four traits (7).

The possibility that the strong correlations among per-unit-mass traits are largely the result of mass normalization of area-proportional traits was discussed in qualitative terms by Field and Mooney (8, 9). This alert has gone largely unnoticed in subsequent studies of leaf traits, perhaps because quantitative estimates of mass versus area proportionality were unavailable until now (Fig. 2). Mass normalization of area-proportional traits induces positive covariance between these traits and negative covariance between each of them and LMA by an amount equal to the variance of LMA [using log-transformed data (7)], which is large in GLOPNET (LMA range: 14 to 1509 g m⁻²). Thus, much of the structure of the mass-based GLOPNET LES is a direct joint result of (i) traits being primarily area-proportional and (ii) high LMA variance, which is a result of the global LL-LMA tradeoff. LL is neither area- nor mass-based, so the strong LL-LMA correlation reported in (1, 2) is unaffected by normalization and likely indicates a robust relationship. The high LMA of long-lived leaves may be necessary to provide the structure and defense needed

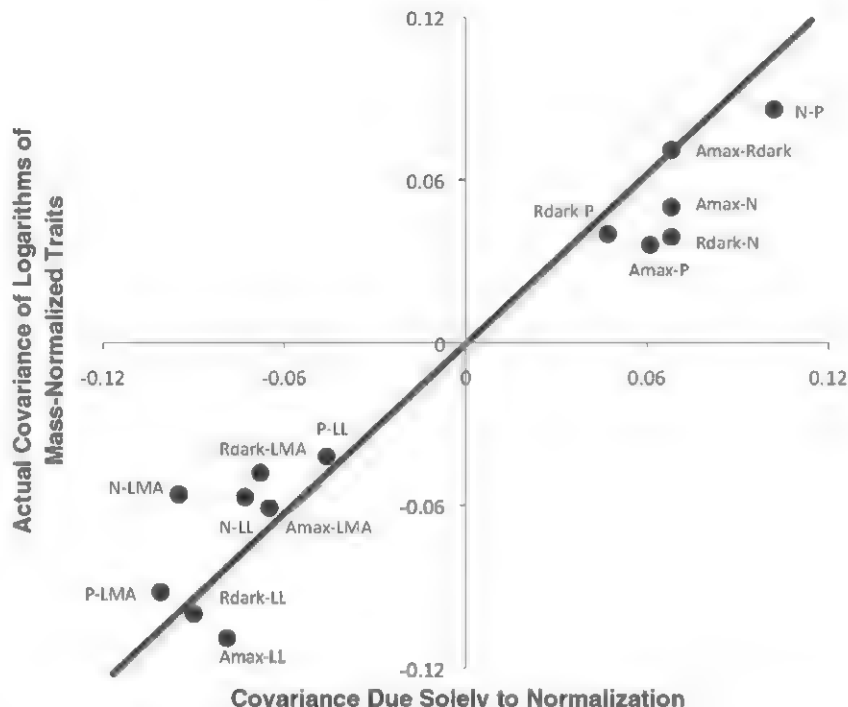


Fig. 3. Actual covariances between logarithms of mass-normalized traits in GLOPNET versus those induced by mass normalization of area-proportional traits. If A_{max} , R_{dark} , N , and P were distributed across species purely proportional to area, then mass normalization would induce the following covariances: $-\text{VAR}(\log_{10}\text{LMA})$ for comparisons involving LMA, $-\text{COV}(\log_{10}\text{LL}, \log_{10}\text{LMA})$ for comparisons involving LL, and $\text{VAR}(\log_{10}\text{LMA})$ for all others (7), where COV is covariance and VAR is variance. Values of $\text{VAR}(\log_{10}\text{LMA})$ and $\text{COV}(\log_{10}\text{LL}, \log_{10}\text{LMA})$ differ for each combination of traits because not all trait values are reported for every species in GLOPNET. Departures of the actual covariances from those predicted to occur solely because of mass normalization are caused by covariances among the minor mass-distributed trait fractions (Fig. 2) and by normalization-independent correlations (figs. S4 and S5).

for long life, which increases nutrient-use efficiency (1, 2, 10–12). Also, because high-LMA leaves are expensive to produce (per unit area) and have similar mean A_{max} per area as low-LMA leaves (1), these high-LMA leaves must live a long time if they are to pay back their construction costs (2, 10). The tradeoff between fast returns on construction costs (low LMA) and long-term returns combined with high nutrient-use efficiency (high LL) likely contributes to the interspecific covariation between LMA and LL (1, 2). Unlike the LL-LMA relationship, most of the covariance between

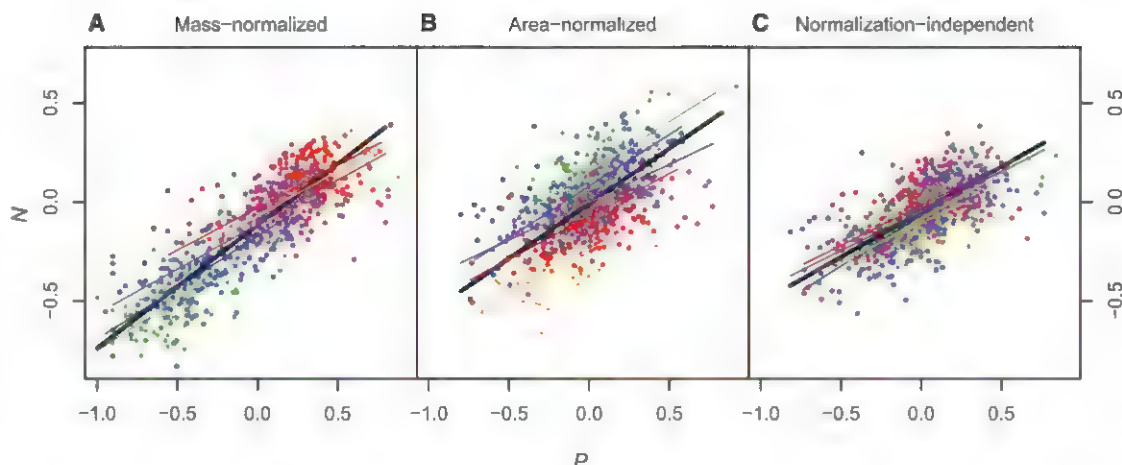
mass-based traits and LL in GLOPNET is due to mass normalization of primarily area-proportional traits that are only weakly correlated with LL (7). The LMA-induced covariances in GLOPNET are quantitatively close to the actual covariances in the mass-normalized LES (Fig. 3), which illustrates the dominance of the covariances created by mass normalization.

To further illustrate the effects of mass normalization, we generated random area-proportional values for A_{max} , R_{dark} , N , and P from statistically independent log-normal distributions with the

Fig. 4. Scatter plots of N versus P from GLOPNET.

(A) Mass-normalized data. (B) Area-normalized data. (C) Normalization-independent values (residuals from Eqs. 1 and 2). Values are $\log_{10}$ -transformed with means rescaled to zero and colored by the $\log_{10}$ LMA z score (number of standard deviations from mean $\log_{10}$ LMA): $z \leq -1$, orange; $-1 < z \leq 0$, red; $0 < z \leq 1$, violet; $1 < z \leq 2$, blue; and $2 < z$, green. Lines show major axis regressions (black, full data set; colored, LMA bins). Slopes of colored lines are not

significantly different from each other or from the black line in (C), but they are significantly different from slopes of black lines in (A) and (B) (7), indicating that the normalization-independent LES is approximately equivalent to sorting species by LMA.



same means and variances as the area-normalized GLOPNET data. We then mass-normalized these random values by dividing them by randomly chosen GLOPNET LMA values (Fig. 1). All correlations in the right-hand panels of Fig. 1 are caused solely by mass normalization and simply reflect the correlation of LMA with either itself or LL. The random values reproduce the general structure in the mass-normalized LES (area-normalized counterpart in fig. S1), even though they lack any functional correlations among $A_{\max}$, R_{dark} , N, and P, such as the physiological dependence of $A_{\max}$ on N (2, 6, 8, 9, 13). Discrepancies between the correlations, slopes, and intercepts of the mass-based LES and those generated from the random LES reflect trait relationships that are not induced by mass normalization and that represent the core of the normalization-independent LES discussed below. Area normalization of randomly generated mass-proportional values produces results unlike the data (fig. S3).

A simple hypothesis explains both the area proportionality of traits and the strong interspecific correlations in the mass-normalized LES in GLOPNET. Evolution has distributed traits associated with photosynthetic function primarily proportional to area, because all leaves are designed to intercept light and capture CO_2 . Regardless of mesophyll depth, only a few cell layers of mesophyll are responsible for the bulk of light capture and photosynthesis (11, 13, 16, 17). However, the large variation in LMA and the strong global LL-LMA relationship imply that plant species vary in their investment in non-photosynthetic functions that increase longevity and require large LMA, such as structural rigidity and defense. Thus, area-normalized light interception, water loss, and photosynthetic traits vary much less than isometrically with LMA. Large variance in LMA reflects variation in ecological conditions that select for different optimal LLs. Together, these factors lead to nearly area-proportional $A_{\max}$, R_{dark} , N, and P; large mass-normalized variance of these traits at any given

leaf area; and thus, large correlations induced by mass normalization.

Dark respiration rate, N, and P are primarily proportional to area because their main function is to support photosynthesis. The significant but minor portion of R_{dark} that is distributed proportional to mass (Fig. 2) likely reflects interspecific variation in GLOPNET in the number and depth of respiring cell layers. The mass-distributed portion of N may be both structural and functional. Every cell wall in a leaf contains nitrogen in structural proteins. Some nitrogen may also contribute to nonphotosynthetic functions, such as carbohydrate dynamics and defense (8). The role of the mass-distributed portion of N and R_{dark} in structural rigidity and defense probably contributes to the strong relationship between LMA and LL. Phosphorus is located primarily in organelles in the cytosol (14) and is therefore not as closely bound to LMA as structural nitrogen. The stoichiometric ratio of N to P increases with LMA in GLOPNET (12) because, on average, mass-proportional fractions are larger for N than for P.

Because normalization-induced covariance scales with the variance of $\log$ LMA, it can be minimized by sorting species into groups with similar LMA. Doing so with GLOPNET reveals that area and mass normalization produce similar trait relationships, both within a given LMA group (compare the slopes of same-colored lines between Fig. 4, A and B) and between groups with different LMA (compare the slopes of lines with different colors within Fig. 4, A and B). The similarity of slopes across LMA groups reflects the approximate multivariate log-normality of the GLOPNET data and points to a new LES defined by the multivariate normal distribution of the logarithms of $A_{\max}$, R_{dark} , N, and P conditional on $\log$ (LMA) (7). Previous analyses included LMA as a covariate when regressing one trait on another (1, 2, 9). Here, we extend this approach to derive a normalization-independent LES for $A_{\max}$, R_{dark} , N, and P that is independent of LMA and

mass or area normalization (7). Consider the following ordinary least-squares (OLS) regressions

$$\log_{10}X_{\text{Aik}} = I_i + S_i \times \log_{10}\text{LMA}_k + n_{ik} \quad (1)$$

$$\log_{10}X_{\text{Mik}} = I_i + (S_i - 1) \times \log_{10}\text{LMA}_k + n_{ik} \quad (2)$$

where $\log_{10}X_{\text{Aik}}$ and $\log_{10}X_{\text{Mik}}$ are, respectively, $\log_{10}$ -transformed area- and mass-normalized values for trait i , species k ; I_i and S_i are intercept and slope parameters for trait i ; and n_{ik} is a normally distributed residual. Subtracting $\log_{10}\text{LMA}_k$ from both sides of Eq. 1 yields Eq. 2. Thus, Eqs. 1 and 2 are mathematically identical: Both regressions yield identical estimates for I_i and S_i and identical values for n_{ik} . Plotting the residuals for two traits against each other shows the normalization-independent relationships (Fig. 4C), which are very similar to those obtained from sorting species into LMA groups (Fig. 4, A and B). Estimates of S_i are consistent with the independently estimated area- versus mass-proportional fractions in Fig. 2 (see comparison in fig. S6), which suggests that the normalization-independent LES accurately captures functional relationships between traits that are altered by area and (especially) mass normalization.

The normalization-independent LES probably reflects universal functional constraints and/or adaptation affecting leaf carbon and nutrient dynamics (2, 11, 15). Pairwise and multivariate trait relationships estimated from the normalization-independent values (i.e., residuals from Eqs. 1 and 2) are usually intermediate in strength between those estimated from area- and mass-normalized values and sometimes have significantly different regression slopes. For example [see also (7)], the standardized major axis regression slope [commonly used in allometry (12)] between mass-normalized N and P in GLOPNET is 0.67, as predicted by metabolic scaling theory (15). However, 2/3 is significantly steeper than the normalization-independent slope of 0.59 (table S2) (7), implying that across the global flora, leaves accrue less N per unit P than predicted by metabolic theory.

The normalization-independent LES has important implications for global vegetation models (5, 6), which include coupled carbon-nitrogen cycles but lack explicit treatment of biodiversity within a small number (roughly 10) of plant functional types (PFTs). Most of these models assume that $A_{\max}$ and R_{dark} are proportional to leaf nitrogen, which appears justified within the vertical canopy gradient of individual plants (5, 13, 16–18). However, much variation in N is the result of differences among species, and less than half of this interspecific variation is explained by global PFT classifications [see figure 5 in (19)]. The assumption that $A_{\max}$ and R_{dark} are proportional to N (log-log slopes of 1) appears reasonably consistent with the log-log OLS slopes of per-unit-mass $A_{\max}$ and R_{dark} versus N (1.25 ± 0.04 and 1.06 ± 0.06 , respectively; OLS regression because changes in N are assumed causal), but these slopes are amplified by hidden changes in LMA (7). If a high-N species replaces a low-N species with the same LMA, then the correct dependencies of $A_{\max}$ and R_{dark} on N are given by the normalization-independent OLS slopes (0.68 ± 0.05 for $A_{\max}$ versus N; 0.66 ± 0.0 for R_{dark} versus N), which control for LMA. The slopes obtained from mass-normalized traits are too steep, implying a carbon-nitrogen feedback that is too strong. In contrast, the carbon-cycle feedback implied by area-based OLS slopes is too weak (0.45 ± 0.04 for $A_{\max}$ versus N; 0.65 ± 0.06 for R_{dark} versus N) because N per area (but not $A_{\max}$ per area) increases with LMA due to its substantial mass-proportional fraction (Fig. 2); this inflates the variance in the independent variable (N) in the regression and reduces the estimated slope (7).

The preceding example assumes that species replacement is associated with increasing N but constant LMA. Because LMA may also change, we now consider the opposite extreme, in which normalization-independent N is held constant and LMA is variable, so that per area increases as LMA increases. In this case, interspecific patterns in GLOPNET imply that an increase in N per area (holding normalization-independent N constant) would be associated with almost no change in $A_{\max}$ and a weak increase in R_{dark} (log-log slopes of $A_{\max}$ per area and R_{dark} per area on N per area, conditional on constant normalization-independent N: 0.06 ± 0.04 and 0.50 ± 0.07 , respectively). Thus, the $A_{\max}$ -N and R_{dark} -N relationships in many global models are again steeper than the interspecific patterns in GLOPNET. Because R_{dark} per area increases more quickly with N per area than $A_{\max}$ per area, net carbon gain per unit leaf area may actually decrease as N per area increases. Intermediate cases involving changes in both LMA and normalization-independent N produce leaf-level $A_{\max}$ -N and R_{dark} -N dependencies that are intermediate between the two extreme cases.

Inconsistencies between the interspecific trait relationships in GLOPNET and the relationships in global vegetation models, and the large amount of within-PFT trait variation in nature (19), imply that global models will need more realistic treatments of biodiversity if they are to predict global

carbon-nitrogen feedbacks. Greater biodiversity could be included in global models by replacing discrete PFTs with a species continuum (2). On the surface, the 15 tight correlations of the mass-normalized LES appear to offer the possibility of capturing 75% of global leaf trait diversity with a single principal component axis (2). However, because much of this visually appealing structure is the result of normalization combined with LMA variation, this single axis excludes important aspects of biodiversity that are orthogonal to LMA. Accurately representing biodiversity in global models will probably require including at least two axes of variation, such as LMA and the first principal component of the normalization-independent LES (7). Alternatively, this normalization-independent axis could be used to represent interspecific variation within the PFTs currently used in global models.

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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 to S6
Tables S1 to S4
References (20–22)

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Early Mesodermal Cues Assign Avian Cardiac Pacemaker Fate Potential in a Tertiary Heart Field

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Cardiac pacemaker cells autonomously generate electrical impulses that initiate and maintain the rhythmic contraction of the heart. Although the majority of heart cells are thought to originate from the primary and secondary heart fields, we found that chick pacemaker cells arise from a discrete region of mesoderm outside of these fields. Shortly after gastrulation, canonical Wnts promote the recruitment of mesodermal cells within this region into the pacemaker lineage. These findings suggest that cardiac pacemaker cells are physically segregated and molecularly programmed in a tertiary heart field prior to the onset of cardiac morphogenesis.

The rhythm of the heart is maintained by a specialized subclass of myocytes known as cardiac pacemaker cells (PCs). These cells generate action potentials (APs) in a cyclic manner to stimulate cardiac contractions. The anatomic position of mature PCs, the sinoatrial node (SAN), was described more than 100 years ago

(1), however, little is known regarding the ontogeny or molecular mechanisms that specify PCs during development. This study was designed to address the timing, location, and mechanisms of PC cell fate acquisition.

Electrophysiological studies (2–4) have mapped cells that initiate cardiac APs to the inflow region at heart tube, looping, and septation stages. However, recent evidence indicates that as the heart matures, it continually expands, with cells being added to both the inflow and outflow segments

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[reviewed in (5)]. To determine which, if any, of the previously identified developmental pacing centers give rise to the mature SAN, we used optical mapping to image the AP initiation site and propagation pattern in embryonic chick hearts (4). Coincident with the heart's first contractions at stage 10 [St10; Hamburger and Hamilton staging (6)], the AP initiation site was preferentially associated with the left posterior inflow segment of the heart [Fig. 1, A (red region) and F]. Left-sided pacing remained dominant through the process of dextral looping (Fig. 1, B and F). By late heart looping (St18), the AP initiation site shifted to the ventral surface of the right inflow, juxtaposed to and outside of the forming atria (Fig. 1C). From this stage on, all hearts displayed a right-sided AP initiation site (Fig. 1, D to F; see also movies S1 to S5).

As the AP initiation site shifted from left to right, AP morphology changed substantially, displaying pronounced slow diastolic depolarization and shorter AP duration than earlier pacing centers (fig. S1). Right-sided pacemakers additionally exhibited a unique expression profile, becoming enriched for genes associated with mature PC AP generation, including *Hcn4*, *Serca2*, and *Ryr2* (7–10), and coexpressing the atrial and ventricular muscle markers *Amhc1* and *Vmhc1* (fig. S2). To determine whether these differences were due to the maturation of migrating earlier pacing cells or were caused by the differentiation of a new cell population, we used vital lipophilic fluorescent dyes to trace the fates of early pacing cells. In no case did dye-labeled cells from left-sided AP initiation sites contribute to older pacing centers (Fig. 1, G and H, and fig. S3). In contrast, cells

from the postlooping right inflow remained associated with the heart's pacing region at all subsequent stages examined, eventually integrating into the SAN region at the back of the right atria (Fig. 1, I and J, and fig. S4). These data demonstrate that PC precursors emerge from a population of cells that are electrically inactive during the initial stages of heart development and begin pacing the heart within about an 8-hour developmental window that coincides with late dextral looping.

To identify when the assignment of PC fate occurs, we determined the location of PC precursors at stages prior to their electrical activation. Because none of the above markers displayed similar enrichment at earlier time points (fig. S2), we used nonmarker, direct cell labeling to create a series of geometric fate maps. Fate maps were scored according to labeled cell incorporation into the late looping stage PC region; scores were verified by whole-mount in situ hybridization for *Hcn4* (figs. S5 and S6). At each of the stages examined, including gastrulation (St5), neurula (St8), and heart tube (St10) stages, PC precursors were mapped to a discrete region of the right lateral plate mesoderm (Fig. 2A). This region was posterior to the classical primary and secondary heart fields, as previously defined by cell tracing experiments and *Nkx2.5* and *Isl1* expression (Fig. 2B) (11–17). Because of the limitations of vital dye labeling, we cannot rule out that at each of these stages other regions contribute to the PCs. However, labeled cells occupied the majority of the available area (fig. S5) when present at the ventral right inflow, so any outside contribution would have to be minor.

PC precursors mapped to a region of the embryo that had not previously been identified as cardiogenic. Given the relatively large distance between PC precursors and the *Nkx2.5* and *Isl1* expression domains currently associated with cardiac mesoderm, we wanted to determine the distribution of cell fates within this region where *Nkx2.5* and *Isl1* were undetectable. A contour plot of the St8 labeling data revealed that the region with the highest probability of PC fate was 100 μ m in diameter and centered 300 μ m lateral to somite 3 (Fig. 2C). The surrounding *Nkx2.5*- and *Isl1*-negative mesoderm generated atria, atrioventricular junction, and the proepicardium (Fig. 2D and fig. S5); this finding indicates that large portions of the cardiogenic mesoderm do not express detectable levels of *Nkx2.5* or *Isl1* at St8. PC precursors did not substantially overlap with adjacent cardiac cell types, which suggests that heart precursors spatially segregate very early during lateral plate mesoderm formation.

Using the above fate mapping information, we sought to determine when PC fate became specified. PC precursors and primary and secondary heart field cells (*Nkx2.5*- and *Isl1*-positive domain) were isolated at the embryonic stages outlined above and allowed to differentiate *ex vivo*. The physiological and molecular identity of explants was then monitored. St18 explants spontaneously initiated APs with a periodicity of 256 ± 29 ms and displayed phase 4 (diastolic) depolarization (Fig. 3, A and B, fig. S7, and movie S7) distinct from that of stage-matched working myocardial explants. PC AP characteristics were not present in St5 explants (Fig. 3, A and B, fig. S7, and movie S7). Explants from St5

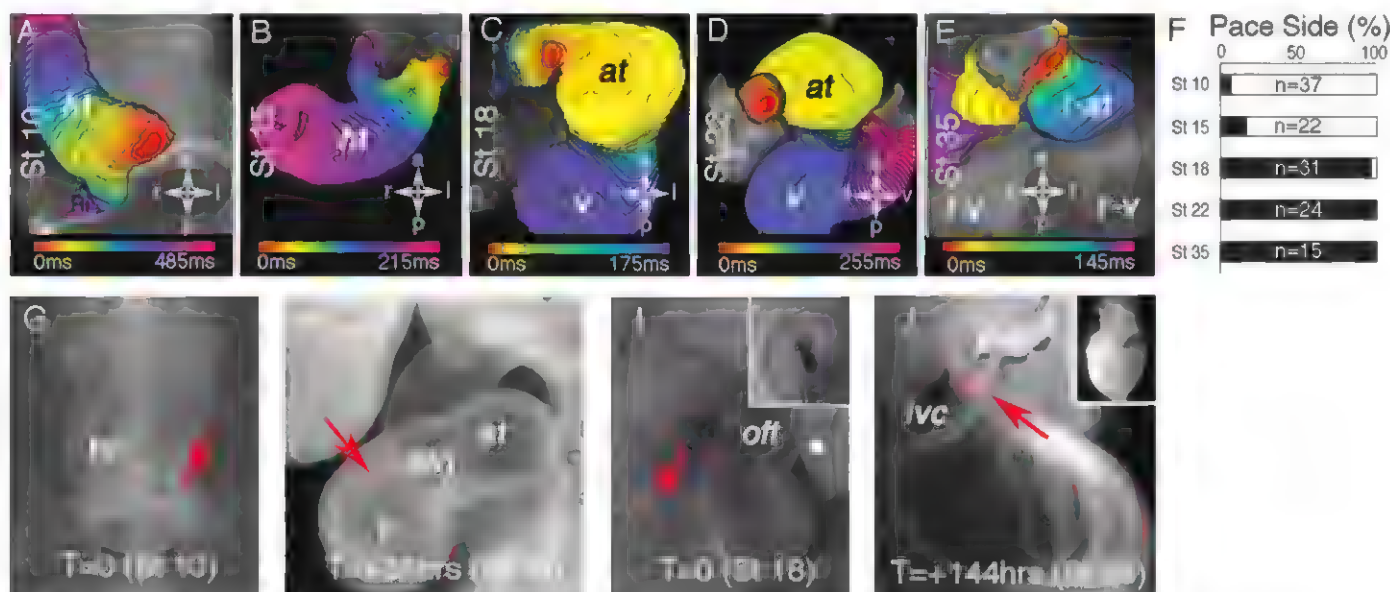


Fig. 1. PCs begin pacing heart rhythm during late looping stages. (A to E) Isochronal maps denoting AP initiation site (red) and propagation pattern from St10 to St35. (F) Pacemaker side spanning five progressive embryonic stages (white, left-sided pacemaker; black, right-sided pacemaker). (G and H) Labeling of St10 AP initiation site (arrow) developed for 36 hours to St18. (I

and J) Labeling of St18 AP initiation site (arrow) developed 144 hours to St35. Insets show low-magnification images of labeled embryo (a, anterior; p, posterior; r, right; l, left; ht, heart tube; v, ventricle; at, atria; avj, atrioventricular junction; r-at, right atria; l-v, left ventricle; r-v, right ventricle; oft, outflow tract; svc, superior vena cava; ivc, inferior vena cava).

had large variations in interbeat intervals and slow beating rates, lacked phase 4 depolarization, and displayed an expression profile inconsistent with St18 PCs (fig. S8). PC precursors from both St8 and St10, however, did differentiate into PC-like cells after 72 hours of culture. Both stages displayed clear phase 4 depolarization, which was absent in age-matched primary and secondary heart field-derived cells (Fig. 3, A and B, fig. S7, and movie S7), and spontaneous depolarization was observed at intervals of 303 ± 53 ms and 294 ± 45 ms, respectively. Expression profiles of explants from St8 were consistent with in vivo PC as described above, showing enrichment for *Hcn4*, *Serca2*, and *Ryr2*, as well as co-expressing *Amhc1* and *Vmhc1* (fig. S8).

These data suggest that by St8, PC fate is already established in the Nkx2.5- and Isl1-negative lateral plate mesoderm. To determine the spatial restrictions of PC specification, we

isolated mesoderm directly adjacent to the PC precursors from the presumptive atria, atrioventricular junction, and proepicardium (see Fig. 3C). Only the PC region displayed elevated phase 4 depolarization and a high rate of AP production (Fig. 3D and fig. S7). These findings suggest that PC fate is specified in a highly restricted subdomain of the right lateral plate mesoderm by St8, and that the initial events dictating the functional divergence of PC fate from the adjacent working myocardium must occur before this stage.

Additionally, our data indicate that a large region of mesoderm outside of the primary and secondary heart fields is already specified into working myocardial and PC fates by St8. To distinguish this mesodermal subdomain from the more classically defined heart fields, we refer to it as a tertiary heart field in chick. Although the precise boundaries of the primary and secondary heart fields remain controversial (18), our high-

resolution fate mapping reveals the distribution and boundaries of several subtypes of cardiac precursors within the tertiary heart field. Conservation of this field in other model systems will require further validation.

Many studies have identified factors necessary for inducing myocyte specification in the primary and secondary heart fields. To determine factors that may play a role in inducing PC fate within the tertiary heart field, we examined the expression of several factors thought to positively or negatively influence myocyte specification during the developmental window outlined above. Expression of a canonical Wnt, *Wnt8c*, was detected in the region of prespecified PCs but not in the more anterior heart fields (fig. S9). Additionally, PC precursors, but not heart field cells, displayed nuclear accumulation of β -catenin suggestive of active Wnt signaling (fig. S9, I to L). We found this surprising because Wnts have previously been

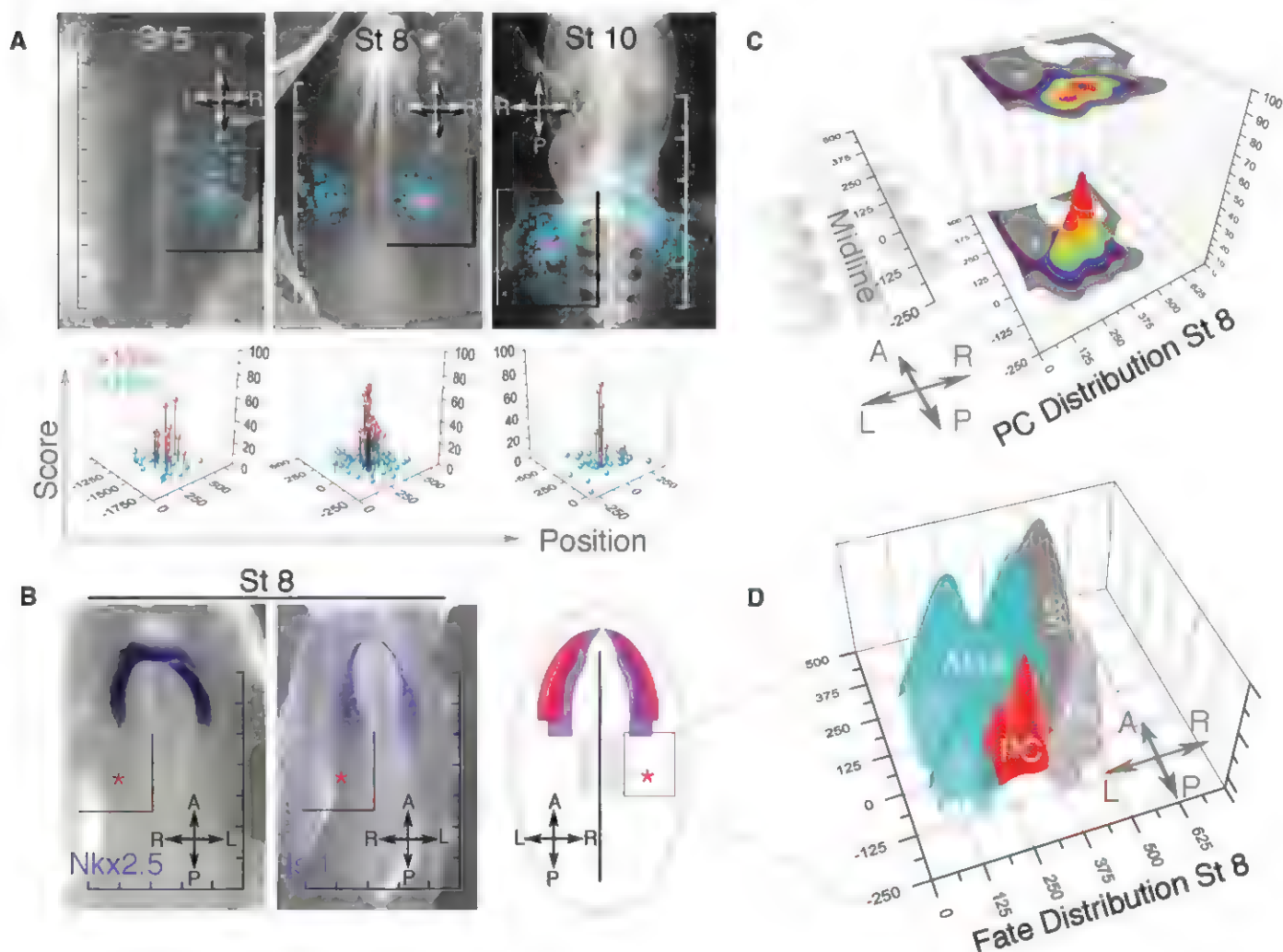


Fig. 2. PCs originate from mesoderm posterior to the heart fields. (A) Fate maps depicting the location of PC progenitors at St5, St8, and St10 (St5 and St8, dorsal view; St10, ventral view). Red data points denote sites with >10% PCs; blue, all other tagged sites. Scale bar, 250 μ m per division. Lower panel: Quantification of labels in the boxed regions (see fig. S5). **(B)** In situ

hybridization (dorsal view) and schematic (ventral view) for heart field markers Nkx2.5 and Isl1 at St8. Asterisks denote PC region. **(C)** Best-fit contour plot of data from St8 fate mapping. **(D)** Overlays of surface plots indicating location of atrial, atrioventricular junction, proepicardial, and pacemaker precursors. A, anterior; P, posterior; R, right; L, left; PE, proepicardium.

Fig. 3. PC fate is specified by St8. (A) Membrane depolarization recorded from heart field. (B) PC precursors isolated from indicated stages. (C) Schematic indicating sites of mesodermal isolation from St8 embryos relative to *Nkx2.5* and *Isl1* expression. HF, heart field; A, atria; AVJ, atrioventricular junction. (D) Representative optical tracings of membrane potential from regions indicated in (C) after 72 hours of culture.

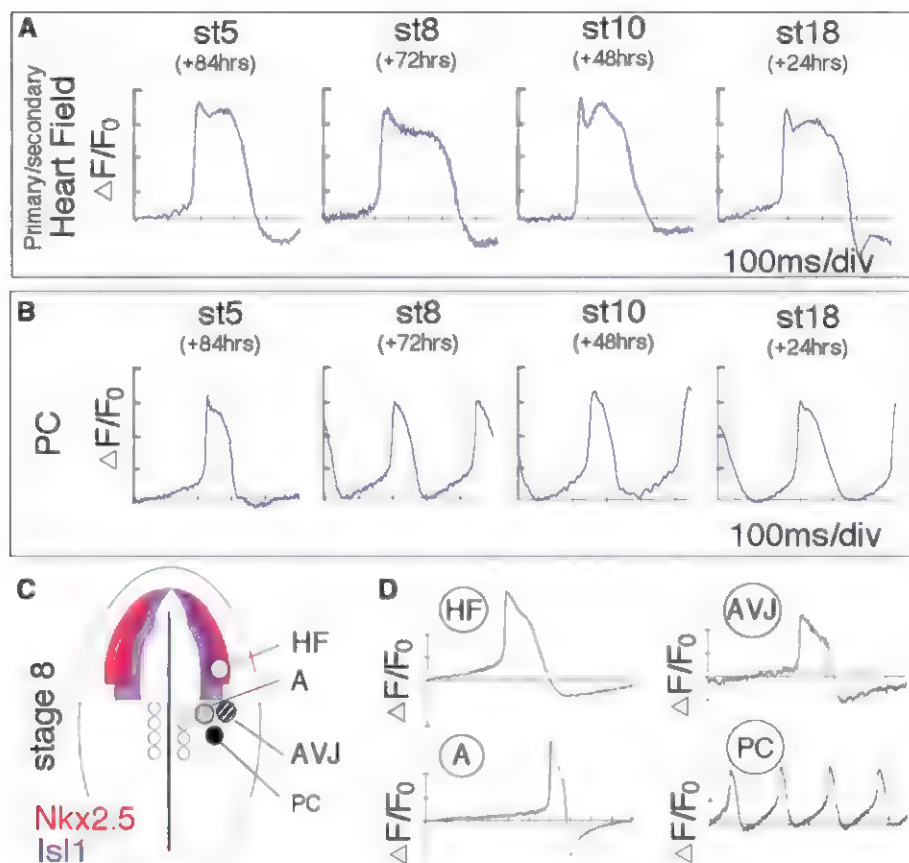
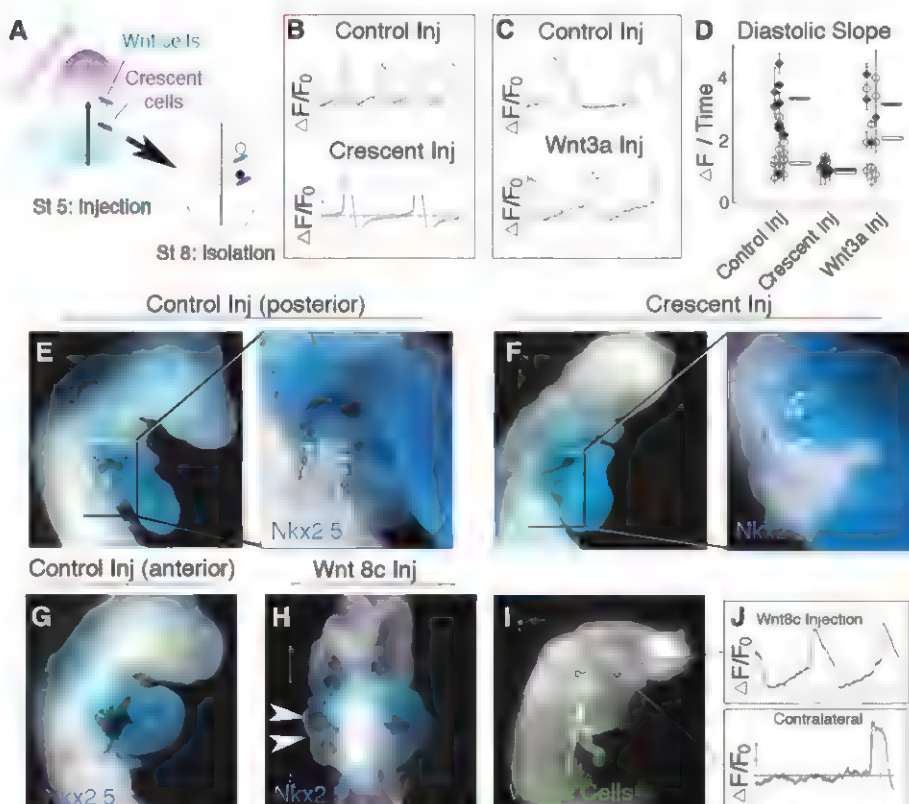


Fig. 4. Canonical Wnt signaling promotes PC fate. (A) Schematic of stage 5 embryo indicating Crescent and *Wnt8c* expression domains. (B and C) After injection of Crescent- or *Wnt*-expressing cells, embryos were developed to St8, when PC or heart field precursors were isolated and placed in culture. Shown are optical membrane potential recordings from PC or heart field cultures after culture. (D) Quantification of diastolic slope from PCs (solid diamonds) or heart field cells (open circles). (E to H) *Nkx2.5* expression is expanded into the PC region after injection of Crescent-expressing cells [dashed area of high-magnification insets from (E) and (F)] and is lost in the heart after *Wnt* cell injection [arrow in (H)] relative to injection of control cells. (I and J) Location of *Wnt8c* expression relative to ectopic PC-like AP generation.



identified as inhibitory for heart field specification (19, 20).

To determine whether Wnt signaling is required to promote PC fate, we microinjected cells expressing the soluble Wnt antagonist Crescent (19, 20) adjacent to PC precursors before their specification. After 8 hours, PC precursors were explanted and allowed to differentiate ex vivo. Exposure to Crescent decreased the slope of PC phase 4 depolarization by 65% relative to control injections (Fig. 4, B and D). These experiments could not rule out the possibility that Crescent is interacting with factors not associated with canonical Wnt signaling. Therefore, to further demonstrate that Wnt signaling was capable of inducing PC fate, we injected Wnt-expressing cells into the presumptive heart fields. This resulted in a 69% increase in phase 4 slope (Fig. 4, C and D). We then used Bio, a pharmacological inhibitor of glycogen synthase kinase 3 (GSK3) that has been shown to stabilize β -catenin (21, 22) to activate Wnt signaling in the heart field. Consistent with the findings above, 10 μ M Bio increased diastolic slope in heart field explants relative to control cells (fig. S10).

When we allowed injected embryos to develop to late looping stages, aberrant Wnt signaling led to severe morphological defects, consistent with previous reports (Fig. 4, F and H) (23). Crescent injection adjacent to PC precursors led to the ectopic expression of Nkx2.5 in PC at St18, which is in agreement with a conversion of PC into a more working myocardial fate (24) (Fig. 4, E and F). About 35% of Wnt-injected embryos survived to heart looping stages. Wnt introduction into the primary and secondary heart field mesoderm resulted in irregularly contracting hearts, with decreased Nkx2.5 expression on the injected

side of the embryo (Fig. 4, G and H). To confirm that these Nkx2.5-negative regions were still electrically active, we performed optical mapping. Consistent with a Wnt-based conversion of working myocardium into PC-like cells, we detected retrograde propagation (outflow toward inflow) as well as ectopic pacemaker sites (movie S8). These ectopic sites were restricted to the Wnt-injected side of the embryo and displayed AP shapes similar to those of control PCs (Fig. 4, I and J, and movie S8).

These findings suggest that early mesodermal Wnt-mediated cues are sufficient to induce pacemaker-like fates that do not manifest until late looping stages. However, Wnts are broadly and bilaterally expressed in the posterior mesoderm, so it is likely that additional cues are required to restrict PC fate, including laterality genes (25, 26). The early diversification of PC fate from the working myocardium suggests that fate specification is assigned directly in the lateral plate mesoderm, and is not the result of the specialization of an already functional embryonic myocyte. These data establish a framework through which PC development should be viewed, thereby providing a foundation for tissue engineering and stem cell-based approaches for PC generation.

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Supplementary Materials

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Movies S1 to S8
References (27, 28)

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Wolbachia Invades *Anopheles stephensi* Populations and Induces Refractoriness to *Plasmodium* Infection

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Wolbachia is a maternally transmitted symbiotic bacterium of insects that has been proposed as a potential agent for the control of insect-transmitted diseases. One of the major limitations preventing the development of *Wolbachia* for malaria control has been the inability to establish inherited infections of *Wolbachia* in anopheline mosquitoes. Here, we report the establishment of a stable *Wolbachia* infection in an important malaria vector, *Anopheles stephensi*. In *A. stephensi*, *Wolbachia* strain wAlbB displays both perfect maternal transmission and the ability to induce high levels of cytoplasmic incompatibility. Seeding of naturally uninfected *A. stephensi* populations with infected females repeatedly resulted in *Wolbachia* invasion of laboratory mosquito populations. Furthermore, wAlbB conferred resistance in the mosquito to the human malaria parasite *Plasmodium falciparum*.

The ability of *Wolbachia* to spread through cytoplasmic incompatibility (CI) (1, 2) and render mosquitoes resistant to a variety of human pathogens (3–6) has instigated the development of *Wolbachia*-based strategies for both suppression and replacement of disease vec-

tor populations (2, 7). Given their medical importance, there have been considerable efforts to extend this approach to anopheline malaria vector mosquitoes, which are not naturally infected by *Wolbachia* spp. (8). Over the past two decades, various attempts to artificially generate stably in-

fecting *Anopheles* spp. have failed, raising concern that the *Anopheles* germ line is inhospitable to *Wolbachia* or that *Wolbachia* infection might cause reproductive ablation in *Anopheles* mosquitoes (6). Studies based on a transient somatic infection have recently indicated that *Wolbachia* can inhibit the development of the malaria parasite in the *Anopheles* mosquito, possibly by stimulating a mosquito antiparasitic immune response (5, 6). These results reinforced the potential of a *Wolbachia*-based intervention for malaria vector control, but only if the bacterium could be made to form a stable association with this mosquito.

Anopheles stephensi is the major vector of human malaria in the Middle East and South Asia. We

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infected *A. stephensi* [Liston strain (LIS)] by embryonic microinjection of the *wAlbB* *Wolbachia* strain derived from *Aedes albopictus* (Houston strain) (9). Cytoplasm was withdrawn from *A. albopictus* embryos and directly injected into the posterior of *A. stephensi* early embryos (1). After oviposition, we used polymerase chain reaction (PCR) to test females (G_0) developed from surviving embryos for *Wolbachia* infection. We observed a stable *wAlbB* infection in one isofemale line (designated LB1) at G_1 with a 100% infection frequency maintained through G_{34} (the last generation assayed thus far). At G_9 , G_{10} , and G_{11} , we randomly selected 20 individuals (10 males and 10 females) from the LB1 cage population and tested them by diagnostic PCR (10). All

individuals ($n = 60$) were infected with *wAlbB* (fig. S1).

The 100% maternal transmission efficiency was also confirmed by fluorescence in situ hybridization (FISH) of LB1 mosquito ovaries showing heavy *wAlbB* infection of all ovarian egg chambers. In the ovaries of the 5-day-old non-blood-fed females, *wAlbB* was mainly found in the oocytes of the egg chambers, with a low-level presence in nurse cells (Fig. 1A). This observation is consistent with a previous model showing *Wolbachia* migration from nurse cells to the oocytes through the ring canals during oogenesis (11). As in *A. albopictus* and the transinfected *Aedes aegypti* WB1 line (1, 12), *Wolbachia* was concentrated in the anterior and posterior part of LB1 mos-

quito oocytes 3 days after a blood meal (fig. S2), indicating that the *wAlbB* distribution pattern in the ovaries is conserved between mosquito species.

Of 8087 eggs resulting from crosses between LB1 males and the naturally uninfected LIS females, only 1.2% (95% confidence interval 0.15 to 2.16) hatched (Fig. 1B), indicating a typical CI pattern. We observed a >50% egg-hatch rate in the other cross types. The egg hatches resulting from LB1 self-crosses (52.4%) were significantly lower than those observed in compatible crosses of wild-type individuals (91.0%; $P < 0.01$, $\chi^2 = 2016.4$). Outcrossing of the LB1 females with LIS males for four generations did not improve the egg-hatch rate, but tetracycline treatment of the outcrossed line increased the rate to $85.9 \pm 5.3\%$ (fig. S3), supporting the hypothesis that the *wAlbB* infection is responsible for the reduced hatch rate.

To assess the ability of the *wAlbB* infection to invade a natural uninfected population, we seeded LB1 females at ratios of 5, 10, and 20% into uninfected LIS cage populations composed of 50 females and 50 males. To promote population replacement, we also released 100 LB1 males at every generation to suppress the effective mating of LIS females. In all populations, *wAlbB* increased to 100% infection frequency within eight generations and remained fixed in subsequent generations (Fig. 1C). These results support the potential for *Wolbachia* to mediate population replacement in a public health intervention strategy. Specifically, the *wAlbB* infection was able to invade and replace the naturally uninfected cytotype within eight generations after an introduction rate as low as 5% and continued inundative release of males at a rate of two times the male population size each generation. These results also raise the challenge in application that large-scale programs for breeding and releasing male infected mosquitoes might be necessary, perhaps in conjunction with short-term intensive mosquito abatement.

A transient *Wolbachia* infection in *Anopheles gambiae* mosquitoes is known to inhibit *Plasmodium falciparum* development (5, 6). To assess the possible anti-*P. falciparum* activity of *wAlbB* in the transinfected LB1 mosquitoes, we fed them on a gametocyte culture, along with LIS

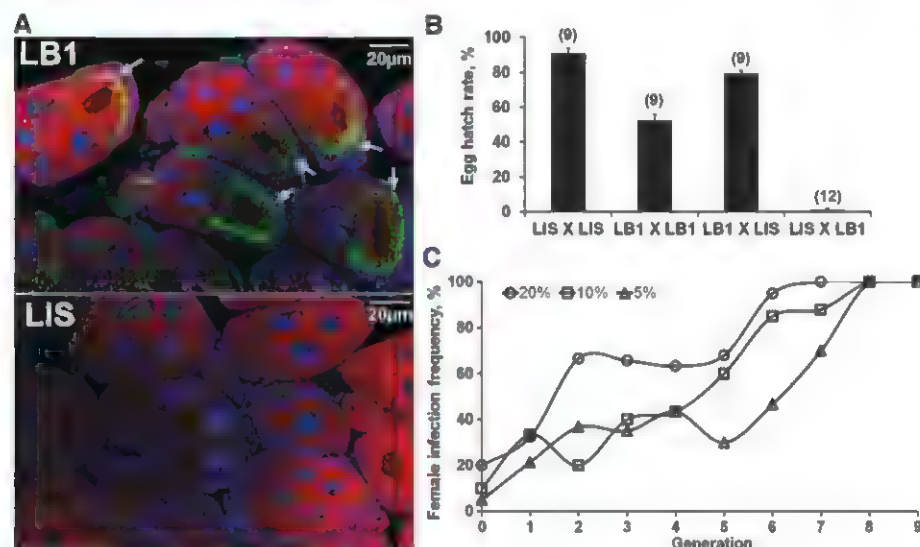
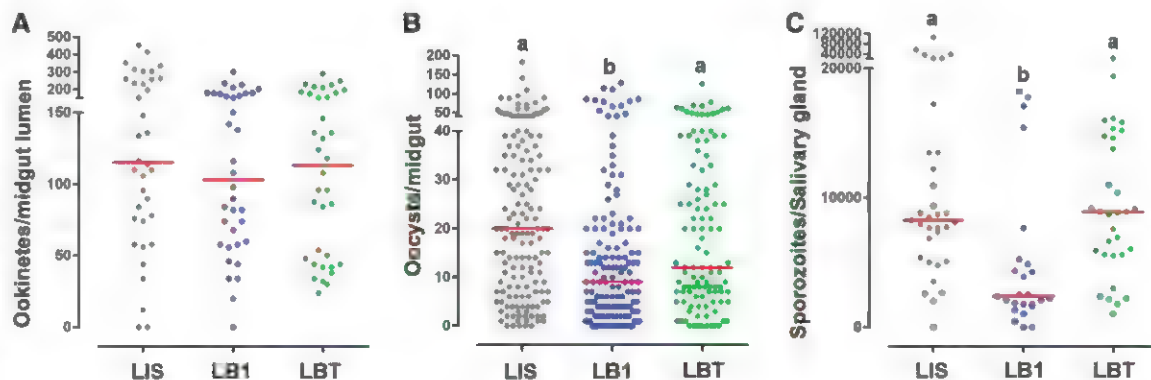


Fig. 1. Establishment and invasion of *Wolbachia wAlbB* in *A. stephensi* populations. (A) *wAlbB* distribution in the ovarian egg chambers of 5-day-old non-blood-fed LB1 females with LIS females as controls. *Wolbachia*, cytoplasm, and nuclear DNA were stained with 16S ribosomal DNA *Wolbachia* probes (green), propidium iodide (red), and 4',6-diamidino-2-phenylindole (blue), respectively. White arrows indicate *Wolbachia*. (B) *wAlbB* induces nearly complete CI in *A. stephensi* when infected males are crossed with uninfected females. Error bars indicate SE. The number of replicates for each of the four cross types is shown in parentheses. (C) *wAlbB* invades the *A. stephensi* laboratory populations. Female infection frequency was measured by PCR after a single release of LB1 females into LIS populations and continued inundative release of LB1 males at a rate of twice the male population size for each generation.

Fig. 2. *Wolbachia wAlbB*-mediated inhibition of *Plasmodium* development.

P. falciparum oocyst (A), oocyst (B), and sporozoite (C) loads in midgut lumens, midguts, and salivary glands, respectively, of *A. stephensi* LIS, LB1, and LBT strains. Points represent the number of parasites from an individual mosquito; horizontal lines indicate the median number of parasites per tissue. Different letters above each column signify distinct statistical groups [(B) $P < 0.0001$ for LB1 versus LIS and $P < 0.01$ for LB1 versus LBT; (C) $P < 0.001$ for both LB1 versus LIS and LB1 versus LBT; Mann-Whitney test].



and the aposymbiotic line LBT mosquitoes (generated by tetracycline treatment of the LB1 strain to remove *wAlbB*) as controls. Although the presence of *wAlbB* had no impact on the ookinete stage parasites before midgut invasion (Fig. 2A and table S1), it did result in a significantly reduced prevalence and mean intensity of the oocyst stage parasite on the basal side of the midgut, as assayed at 7 days postinfection (dpi). Specifically, the LB1 strain displayed significantly lower infection prevalence and intensity than the LIS strain (Mann-Whitney U test, $P < 0.0001$) and the aposymbiotic LBT strain (Mann-Whitney U test, $P < 0.01$), whereas no difference was observed between the LIS and LBT strains (Fig. 2B and table S1). We also investigated the impact of *wAlbB* on the salivary gland sporozoite stage infection at 14 dpi and observed a greater inhibition than at the oocyst stage (Fig. 2C and table S1). *wAlbB* infection resulted in a 3.4- and 3.7-fold reduction in the sporozoite loads in salivary glands of LB1 mosquitoes when compared with LIS and LBT mosquitoes, respectively (Mann-Whitney U test, $P < 0.001$) (Fig. 2C and table S1). These data suggest that *wAlbB* inhibit *P. falciparum* development between the preinvasion luminal ookinete and oocyst stages and between the oocyst and salivary gland sporozoite stages.

A local distribution of *wAlbB* and *wMelPop* strains in mosquito somatic tissues, especially those in which pathogens replicate, develop, and travel, is important for *Wolbachia* to induce pathogen interference (4, 13). We examined the *wAlbB* density in midguts, salivary glands, and fat bodies from 7-day-old LB1 non-blood-fed females by real-time PCR. We detected *Wolbachia wAlbB* in all tissues, with a marked 5.9-fold higher density in the fat bodies than in the ovaries (Fig. 3A).

Salivary glands and ovaries contained similar levels of *wAlbB*, whereas midguts had lower infection than ovaries, a distribution confirmed by FISH assay (Fig. 3B). This result is similar to observations made in the transiently infected *A. gambiae*, in that *Wolbachia* resided primarily within cells of the fat bodies and had a low affinity for midgut cells (6).

We have previously shown that *wAlbB* induces the production of reactive oxygen species (ROS) in *Aedes* (14), and other work has shown that ROS can inhibit *Plasmodium* infection in *Anopheles* (15, 16). To explore whether *wAlbB*-induced ROS could play a role in the mosquitoes' resistance to *Plasmodium*, we compared the levels of H_2O_2 in midguts, fat bodies, and whole bodies of LB1 and LIS mosquitoes. The levels of H_2O_2 were significantly higher in tissues of LB1 mosquitoes than in those of LIS mosquitoes (Student's *t* test, $P < 0.01$) (Fig. 4) and nearly twofold higher in whole LB1 than in LIS mosquitoes.

In conclusion, we show that the *Wolbachia wAlbB* strain can form a stable symbiosis with *A. stephensi*, invade laboratory mosquito populations through CI, and confer elevated resistance to *Plasmodium* infection, potentially through ROS generation. Previous failures in establishing a stable *Wolbachia* infection in *Anopheles* mosquitoes may be due to the *Wolbachia* strains used. To form a symbiosis, the *Wolbachia* strain should be sufficiently invasive to establish an infection in germ tissues but without being lethal to the host. The success of *wAlbB* may be attributed to its ability to confer a fitness advantage to its host (10) and its high infectivity to *Anopheles* germ tissues (17). We used a previously described embryo microinjection technique (1) but observed a lower survivor rate, possibly due to the greater sensitivity of *Anopheles* eggs to desiccation. The

low egg-hatch rate associated with the *wAlbB* infection in *A. stephensi*, which is not observed in *wAlbB*-infected *A. aegypti*, may be related to the use of mouse (an unnatural host) blood in this study. A previous study has reported suppression of egg hatch after a long-distance transfer of *wMelPop* into *A. aegypti* feeding on nonhuman blood sources, but only mild decreases when the mosquitoes fed on human blood (18).

The recent success of a field trial has demonstrated that *Wolbachia* can be deployed as a practical dengue intervention strategy, with the potential for area-wide implementation (2). The design of *Wolbachia*-based malaria control strategies would have to accommodate the fact that *Plasmodium* is vectored by multiple and frequently sympatric *Anopheles* species in different parts of the world (19, 20). However, this complication can be resolved by integrating a *Wolbachia*-based approach with other vector control strategies and by targeting the dominant malaria vectors that are the most difficult to control. For example, *Wolbachia* could be used to target outdoor-biting and -resting species that can evade current vector control methods, such as insecticide-treated nets and residual insecticide sprays (21). In our studies, we used a laboratory *P. falciparum* infection model that results in unnaturally high infection intensities, reaching a median of 20 oocysts per midgut, whereas infection levels in nature rarely exceed 2 to 3 oocysts (22). As we have shown in other studies comparing natural and laboratory infection intensities (23), it is quite likely that a stable *wAlbB* infection would confer complete refractoriness under natural field conditions. Our success in rendering *A. stephensi* resistant to *P. falciparum* by stable introduction of *wAlbB* offers a potential approach to permanently reduce the vectorial capacities of dominant malaria vectors in sub-Saharan Africa, one of the most challenging goals in current malaria vector control (21). However, it is still unknown whether *Plasmodium* will develop resistance to ROS or other *Wolbachia*-mediated inhibitory mechanisms in mosquitoes.

Fig. 3. *Wolbachia wAlbB* distribution in somatic tissues of LB1 mosquitoes (G₂₇). (A) The genome copy of *Wolbachia* surface protein (WSP) was measured by real-time PCR and normalized by *A. stephensi* ribosomal protein S6 (RPS6). Different letters above each column signify distinct statistical groups ($P < 0.05$ for comparison between a, b, and c; Student's *t* test). Error bars indicate SEM of at least 10 biological replicates. (B) *Wolbachia wAlbB* distribution in fat body, midgut, and salivary gland of an LB1 mosquito, assayed by FISH as described in Fig. 1A. White arrows indicate *Wolbachia*.

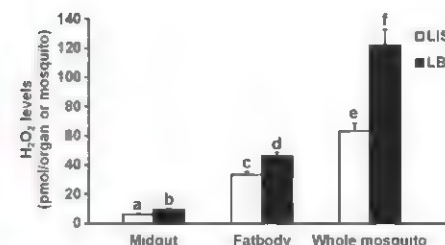
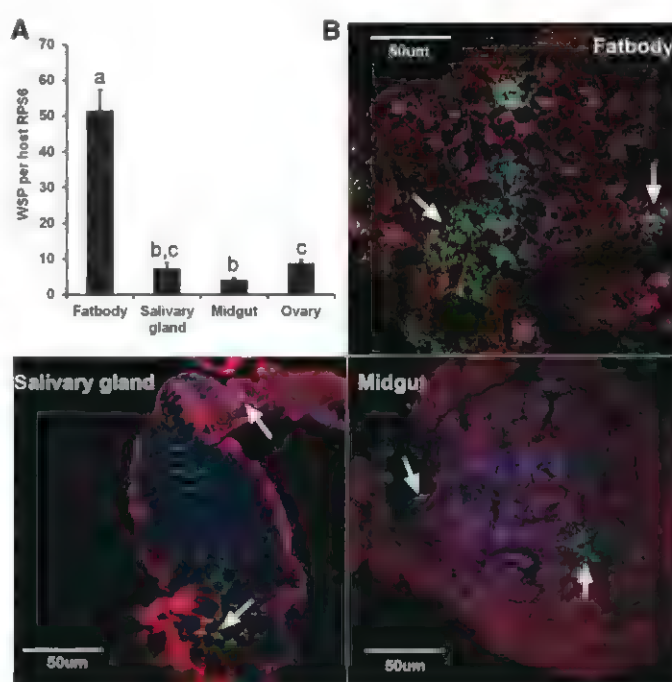


Fig. 4. *Wolbachia*-induced ROS production in LB1 mosquitoes. This figure shows a comparison of H_2O_2 levels in the fat body, midgut, and whole mosquito in 7-day-old LB1 and LIS females before a blood meal. The data shown are means of 6 (fat body and midgut) or 10 (whole-mosquito) replicates. Different letters above each column signify distinct statistical groups ($P < 0.01$ for each pair of comparison between a, b, c, d, e, and f; Student's *t* test). Error bars indicate SEM.

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Supplementary Materials

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Delineating Antibody Recognition in Polyclonal Sera from Patterns of HIV-1 Isolate Neutralization

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Serum characterization and antibody isolation are transforming our understanding of the humoral immune response to viral infection. Here, we show that epitope specificities of HIV-1-neutralizing antibodies in serum can be elucidated from the serum pattern of neutralization against a diverse panel of HIV-1 isolates. We determined “neutralization fingerprints” for 30 neutralizing antibodies on a panel of 34 diverse HIV-1 strains and showed that similarity in neutralization fingerprint correlated with similarity in epitope. We used these fingerprints to delineate specificities of polyclonal sera from 24 HIV-1-infected donors and a chimeric simian-human immunodeficiency virus-infected macaque. Delineated specificities matched published specificities and were further confirmed by antibody isolation for two sera. Patterns of virus-isolate neutralization can thus afford a detailed epitope-specific understanding of neutralizing-antibody responses to viral infection.

Upon infection or vaccination, the adaptive immune system typically generates polyclonal antibody responses that recognize multiple epitopes (1–3). The serologic characterization of such polyclonal responses can inform vaccine design by elucidating which epi-

topes on the antigen are immunodominant and/or targets of pathogen-specific neutralizing antibodies. Such serologic analysis can further lead to the isolation of new monoclonal antibodies that may be of therapeutic value. As a result of extensive effort to understand the antibody response to viral infection, recent years have seen a surge in the isolation of monoclonal antibodies against HIV-1, influenza, hepatitis C, and other viruses (4–15). The link between polyclonal sera and component monoclonal antibodies, however, remains complex and difficult to decipher, in part, because of the extraordinary diversity of circulating antibodies. Viral genetic diversity can be an integral mechanism of immune evasion (16–22); this same diversity may, however, also provide a means by which to understand antibody responses (23, 24). Specifically, monoclonal antibodies targeting the same epitope on an antigen are likely to be affected in a similar way by diversity in that epitope region. When presented with a diverse set

of viral isolates, monoclonal antibodies may thus exhibit characteristic neutralization patterns or “neutralization fingerprints” (Fig. 1). Furthermore, neutralization patterns of a polyclonal serum could be viewed as the combined effect of the neutralization fingerprints of component monoclonal antibodies, and, if this relationship could be deconvoluted, then serum neutralization would serve as a predictor of component-antibody specificity.

To test this conjecture, we selected HIV-1 because of its high viral sequence diversity, the availability of well-characterized sera and antibodies, and the limited number of sites of vulnerability targeted by neutralizing antibodies on the HIV-1 spike (Env). These sites encompass the CD4-binding site (CD4bs), a variable loop V1/V2 site, and a glycan-V3 site on glycoprotein gp120, and the membrane-proximal external region (MPER) on gp41 (4–7, 13, 14, 25–35). The same site of vulnerability may encompass multiple epitopes and, as a result, can be targeted by antibodies with diverse specificities. To determine whether the neutralization fingerprints of HIV-1 monoclonal antibodies are a reflection of their epitope specificities, we utilized neutralization data for a panel of 34 diverse HIV-1 isolates (table S1), for 30 monoclonal antibodies recognizing diverse epitopes on HIV-1 Env, and for two variants of the CD4 receptor (table S2). Neutralization fingerprints for antibodies known to target similar epitopes correlated significantly better (Spearman correlation) than fingerprints of antibodies targeting different epitopes (fig. S1). On the basis of the neutralization-correlation values, antibodies were grouped into 10 clusters (Fig. 2A) (36), by using a clustering cutoff chosen to agree with known antibody structures and epitope-mapping (4–6, 13–15, 25–27, 37–40, 41). Two antibodies, 8ANC195 and HJ16, whose precise epitopes are currently unknown, clustered separately (5, 15), whereas all of the other antibody clusters could be mapped to known sites of Env vulnerability.

Overall, neutralization fingerprints appeared to exhibit sufficient specificity to successfully distinguish between antibodies targeting different

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epitopes on the same overall site of vulnerability (42). The MPER site was recognized by two antibody clusters, each consisting of two antibodies (2F5/m66.6 and 10E8/4E10); this agreed with known MPER-antibody epitopes (fig. S2) and demonstrated the dominant contribution of the recognized epitope, rather than particulars of antibody orientation, to the antibody-neutralization fingerprints: 10E8 and 4E10 recognize the same epitope but use substantially different antibody heavy- and light-chain orientations (13, 43). The V1/V2 site was recognized by a single cluster of PG9-like antibodies. The glycan-V3 site was recognized by two clusters of antibodies: PGT128-like antibodies that target a glycopeptide-epitope and antibody 2G12 that targets a cluster of glycans including N332 (44) on the gp120 surface (25, 26, 45). Last, the CD4bs was targeted by at least three clusters, including those of antibody b12, CD4, and the VRC01-like antibodies (27, 38).

VRC01-like antibodies represented the largest fraction in the analyzed data set and formed the largest antibody-neutralization cluster; they could be grouped around VRC01, a prototypical broad and potent CD4bs antibody capable of neutralizing 90% of circulating HIV-1 strains (4, 27). The VRC01-like cluster included antibodies known to target similar epitopes on gp120, as confirmed by antibody structures in complex with gp120 (VRC01, VRC03, VRC-PG04, and NIH45-46) and epitope-mapping experiments (4, 5, 14, 27, 40). Although published epitope maps suggest that antibody VRC06 recognizes an epitope composed of both CD4- and co-receptor-binding sites (46), the cocrystal structure of VRC06 in complex with core gp120 revealed a similar mode of gp120 recognition by VRC06, as compared with both VRC01 and VRC03 (Fig. 2B), consistent with the similarity observed for the neutraliza-

tion fingerprints of these antibodies (47). These results indicate that clustering of antibodies based on neutralization fingerprints can be an accurate delineator of antibody-epitope specificity.

To assess whether the neutralization signal from polyclonal sera could be deconvoluted into component-antibody specificities, we analyzed neutralization data for sera from HIV-infected donors (48). The neutralization fingerprints for all antibodies within a given cluster were used to create a single representative neutralization fingerprint for the cluster, resulting in a reference set of 10 epitope-specific neutralization fingerprints, one for each antibody cluster (fig. S3) (41). The neutralization pattern for each serum was taken to be a linear combination of the reference-set fingerprints, resulting in an estimate of the relative contribution to serum neutralization (i.e., neutralization prevalence) of the respective component-antibody specificities as described in (41) and Fig. 1.

For most sera, multiple specificities were predicted (Fig. 3)—as might be expected from the polyclonal nature of sera (1–3). To evaluate the neutralization-based serum-epitope predictions, we compared them with component-antibody epitopes determined by other experimental means. We first compared predictions for which serum-neutralization specificities were already known through antibody isolation or extensive epitope mapping. The neutralization-based prediction for donor 45, the source of VRC01 and other VRC01-like antibodies (4), indicated a strong signal for VRC01-like antibodies (Fig. 3A) (49). The prediction for donor N152, the source of antibody 10E8 (13), showed a dominant signal for the 10E8-like cluster (Fig. 3A). Furthermore, longitudinally sampled sera from donor CAP256 showed a PG9-like signal at each of four time points, both by extensive experimental epitope mapping (50) and computational predictions

(Fig. 3B). Last, sera from a chimeric simian-human immunodeficiency virus (SHIV)-infected macaque showed a dominant PGT128-like signal at each of four time points (Fig. 3C), in agreement with published epitope-mapping experiments (51). The neutralization-based method for delineating component-antibody epitopes was thus successful in identifying major antibody responses for sera with confirmed specificities.

Next, we applied our method to prospectively delineate specificities for sera from 21 HIV-1-infected donors (52) and compared the results to (standard) experimental serum mapping data (Fig. 3D): Published assays were used to map serum antibody specificity toward the CD4bs, V1/V2, glycan-V3, and MPER regions (41, 53). The degree of correspondence between the neutralization-based prediction method and standard mapping varied for different sera, with discrepancies partly attributable to known limitations in current standard mapping assays (54) (table S4). Nonetheless, the agreement between the two methods was reasonably good, with at least one of the top two neutralization-delineated specificities identified by standard mapping in ~85% of the tested sera (Fig. 3E) (55).

To further validate our predictions, we selected two sera with different predicted specificities and prevalence levels: (i) serum from donor 127/C predicted to have a dominant VRC01-like specificity with a prevalence level of 0.60 and (ii) serum from donor N27 predicted to have a PGT128-like specificity with a prevalence level of 0.33 (Fig. 3D). We cloned CD4bs antibodies from donor 127/C peripheral blood mononuclear cells (PBMCs) by using an antigen-specific protein probe (RSC3), as described previously (4). We identified two somatic variants, VRC23 and VRC23b that showed strong similarity to other VRC01-like antibodies by germline-gene usage,

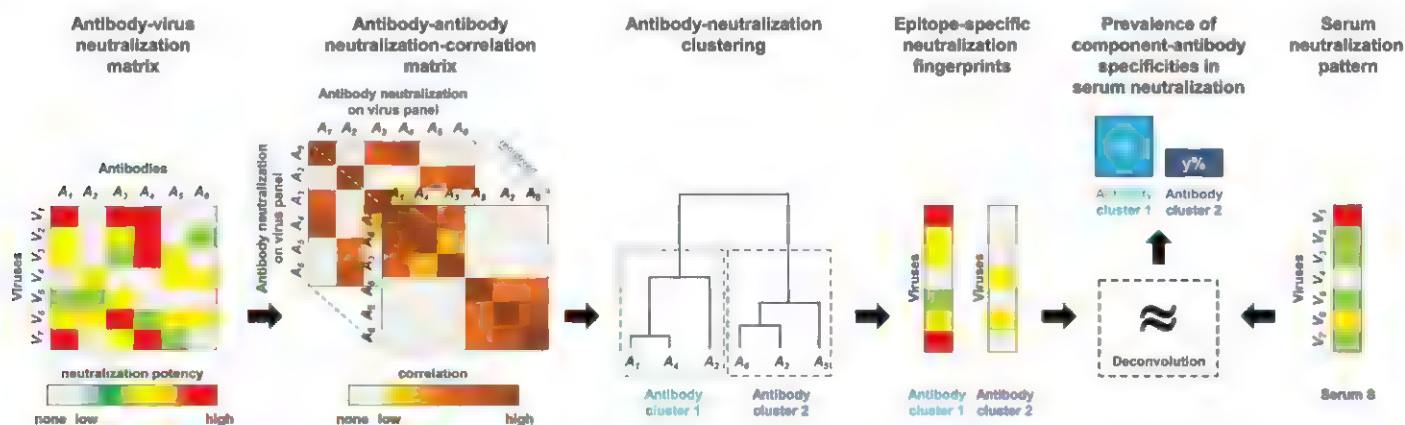


Fig. 1. Definition of antibody neutralization fingerprints and determination of antibody specificities from serum patterns of neutralization. The neutralization fingerprint for an antibody on a panel of diverse viruses may contain sufficient information to define both antibody epitope and molecular specificities of recognition. Here, we show how information from a neutralization matrix can be transformed to delineate epitope specificity. First, a database of neutralization fingerprints for known antibodies can be constructed on the basis of clustering of antibody-neutralization behavior: The neutralization fingerprints for

antibodies (columns: A_1 to A_6) are shown in a neutralization matrix (first (leftmost) panel), with colors representing neutralization potencies against specific viruses (rows: V_1 to V_7); the correlations between antibody neutralization fingerprints (second panel) can help form epitope-specific antibody clusters (third panel). The resultant epitope-specific neutralization fingerprints (fourth panel) can be used to interrogate patterns of neutralization from polyclonal sera. Deconvolution of the serum-neutralization pattern (last panel) into epitope-specific fingerprints identifies epitope specificity of the component antibodies (fifth panel).

sequence signature, binding characteristics, and neutralization (Fig. 4A and figs. S4 to S7) (41). Furthermore, the crystal structure of VRC23 in complex with gp120 revealed substantial similarities with the mode of gp120 recognition by VRC01 and other VRC01-like antibodies (56), confirming the neutralization-based prediction of VRC01-like antibody specificity in serum 127/C. By comparison, standard experimental serum mapping showed borderline evidence for CD4bs activity, with RSC3-based inhibition of 127/C neutralization reaching only 20% (fig. S11). For donor N27, standard serum mapping (Fig. 3D) indicated the presence of glycan-reactive antibodies. We used single B cell culture (5, 13, 41) and recovered one neutralizing antibody, VRC24

(fig. S4). Binding and neutralization assays for VRC24 suggested a glycan-V3 epitope (figs. S12 to S14), with general similarity to (but also some differences from) other antibodies in the PGT128-like cluster. Thus, for both donors 127/C and N27, we successfully confirmed the existence of antibodies with specificities predicted by the neutralization-based method. Furthermore, the neutralization fingerprints for antibodies VRC23 and VRC24 clustered appropriately—within the VRC01-like cluster for VRC23 and most closely to (though not fully intermingled with) the PGT128-like cluster for VRC24 (Fig. 4B)—which demonstrated that, in addition to delineating serum specificity, neutralization fingerprints allow for prospective prediction of monoclonal antibody epitopes.

Analysis of sera from HIV-1-infected donors and, more recently, from SHIV-infected macaques is revolutionizing our understanding of the ability of the humoral immune system to recognize and to neutralize HIV-1 (29–34). Such analysis generally begins with an assessment of serum neutralization on small or moderately sized virus panels (29, 57) and then proceeds with epitope mapping and monoclonal antibody isolation. The ability to delineate component-antibody neutralization specificities directly from serum-neutralization data potentially transforms this process, by providing a computational shortcut for procedures of standard epitope mapping, monoclonal antibody isolation, and crystal structure determination of the epitope (58). Such a

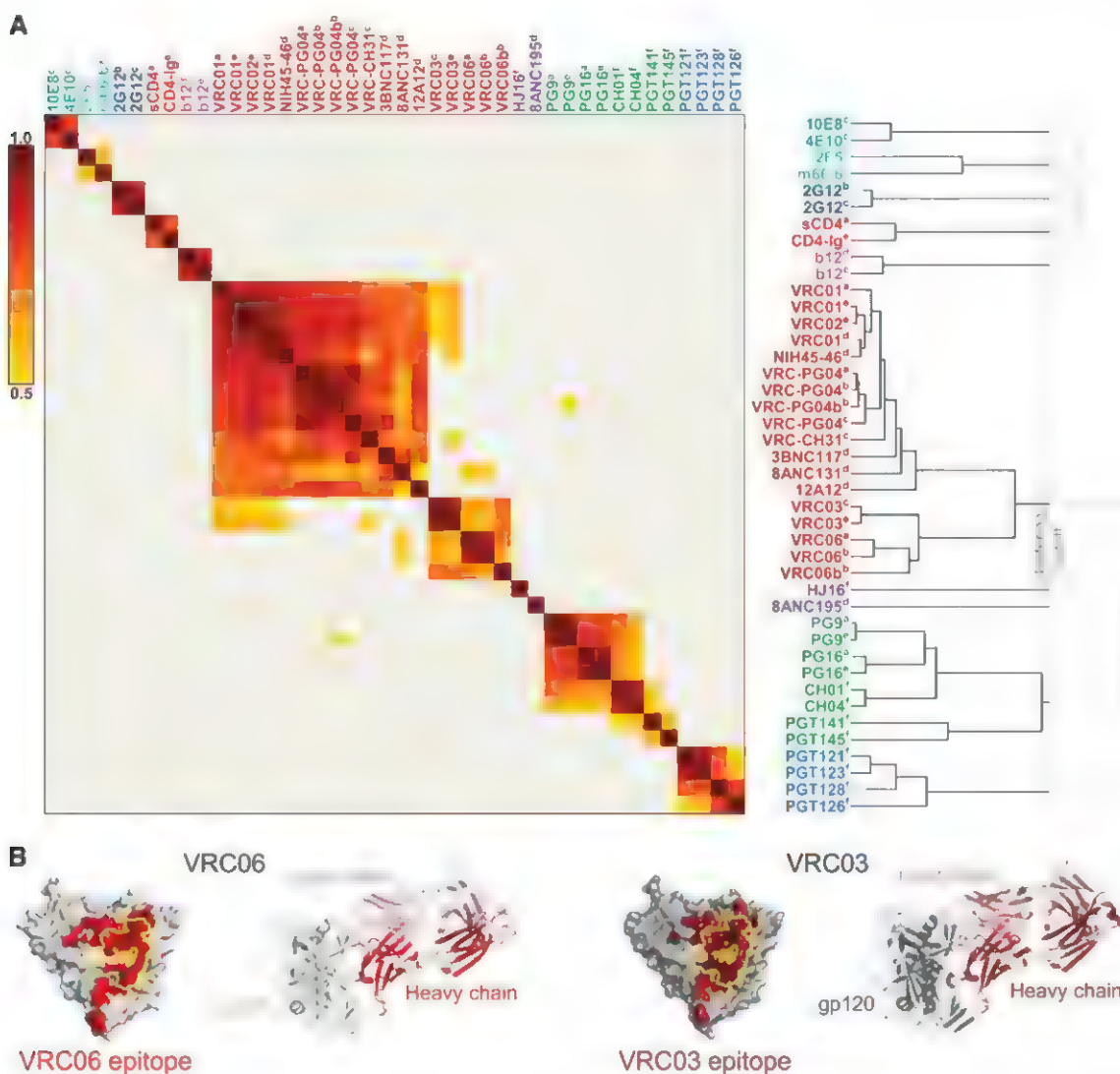


Fig. 2. Neutralization-based clustering of HIV-1 antibodies. (A) Antibody-antibody neutralization-correlation matrix. A set of HIV-1–neutralizing antibodies along with two variants of CD4 were assessed for neutralization on a panel of 34 diverse HIV-1 isolates. Input from six neutralization panels (tables S5 and S6) were used to define this correlation matrix and are indicated by superscripts a through f next to each antibody name, with several antibodies tested on multiple panels. Correlation results are shown in heat-map representation from 0.5 (yellow) to 1.0 (dark brown) and were used as input for antibody clustering. The intersection

of the dotted vertical line with horizontal lines in the clustering tree defines the 10 antibody clusters: VRC01-like (maroon), PG9-like (green), PGT128-like (blue), 2F5-like (light blue), and 10E8-like (aqua), as well as separate clusters for b12, CD4, 2G12, HJ16, and 8ANC195. Antibody order was based on the clustering tree. (B) Comparison of gp120 recognition by antibodies VRC03 and VRC06, shown as epitope and 90 degree-rotated complex. Although VRC03 and VRC06 display substantial phenotypic differences (46), their neutralization fingerprints are highly correlated, and cocrystal structures reveal the same gp120 mode of recognition.

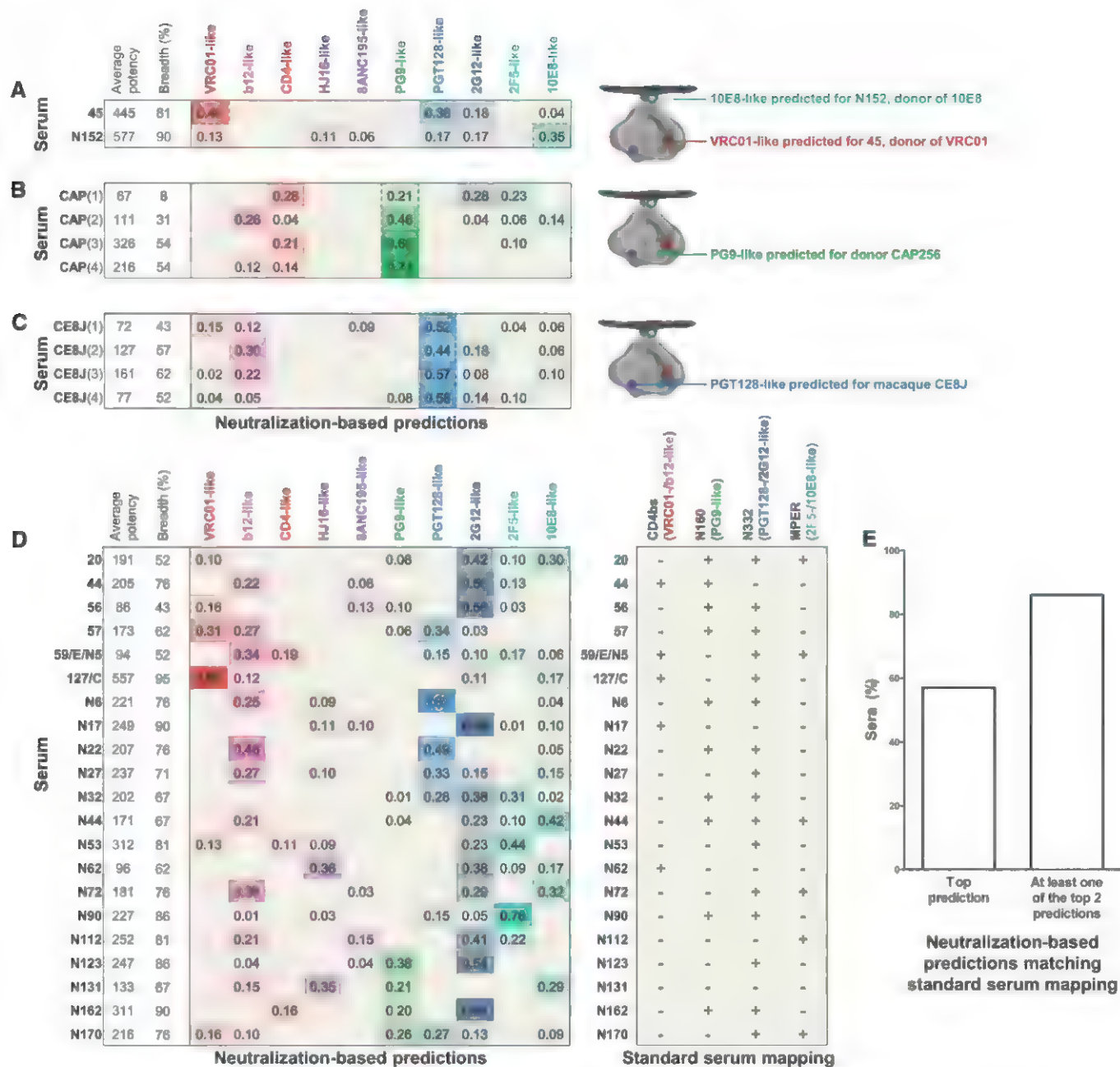


Fig. 3. Delineation of antibody specificities in HIV-1 sera with broadly neutralizing activity. For each serum, the predicted relative prevalence of the different reference-set antibody clusters (colored as in Fig. 2) is shown as a heat map, with darker intensity (higher fractional number) corresponding to a stronger neutralization signal (predicted prevalence level) by the respective antibody cluster. Numbers in each row add up to 1.00, with numbers less than 0.01 not shown. Serum neutralization breadth is shown for the specific virus panels used, and average potency provided as geometric mean inhibitory dilution (ID₅₀). Predictions for (A) serum from donor 45 (the source for antibody VRC01) and from donor N152 (the source for antibody 10E8), (B) sera from four different time points (years 1 to 4) postinfection from donor CAP256, and (C) sera from four different time points (weeks 40,

61, 81, and 100) postinfection from macaque CE8J. To the right of (A) to (C) are schematics of the putative locations on the HIV-1 Env trimer of the epitopes identified in the neutralization-based predictions and previously confirmed to be targeted by antibodies in the sera. (D) Neutralization-based predictions (left) and standard experimental mapping data (right) for an additional set of 21 sera (rows) and 10 epitopes (columns). Epitope groups (bold) for the experimental mapping data were defined on the basis of the assays used; the respective closely matching neutralization-based epitope clusters were grouped accordingly (shown in parentheses). Epitope groups are marked with a plus sign (+) if predicted by the mapping assays to be present in a given serum or with a “-” otherwise. (E) Concordance between neutralization-based and standard serum mapping.

short-cut should afford a more rapid and less arduous delineation of serum component–antibody epitopes and may be especially useful for studies in which sample volumes are limited. Two developments have been key to the success of the

method and are likely to work synergistically to improve such neutralization-based delineation. First, application of robotic technology to neutralization assessment has made data from HIV-1–neutralization panels easier to obtain

(14, 57, 59, 60). Second, advances in monoclonal antibody identification have allowed for the recent isolation of many effective HIV-neutralizing antibodies, and structural analysis has delineated their sites of recognition (20, 35, 61); although

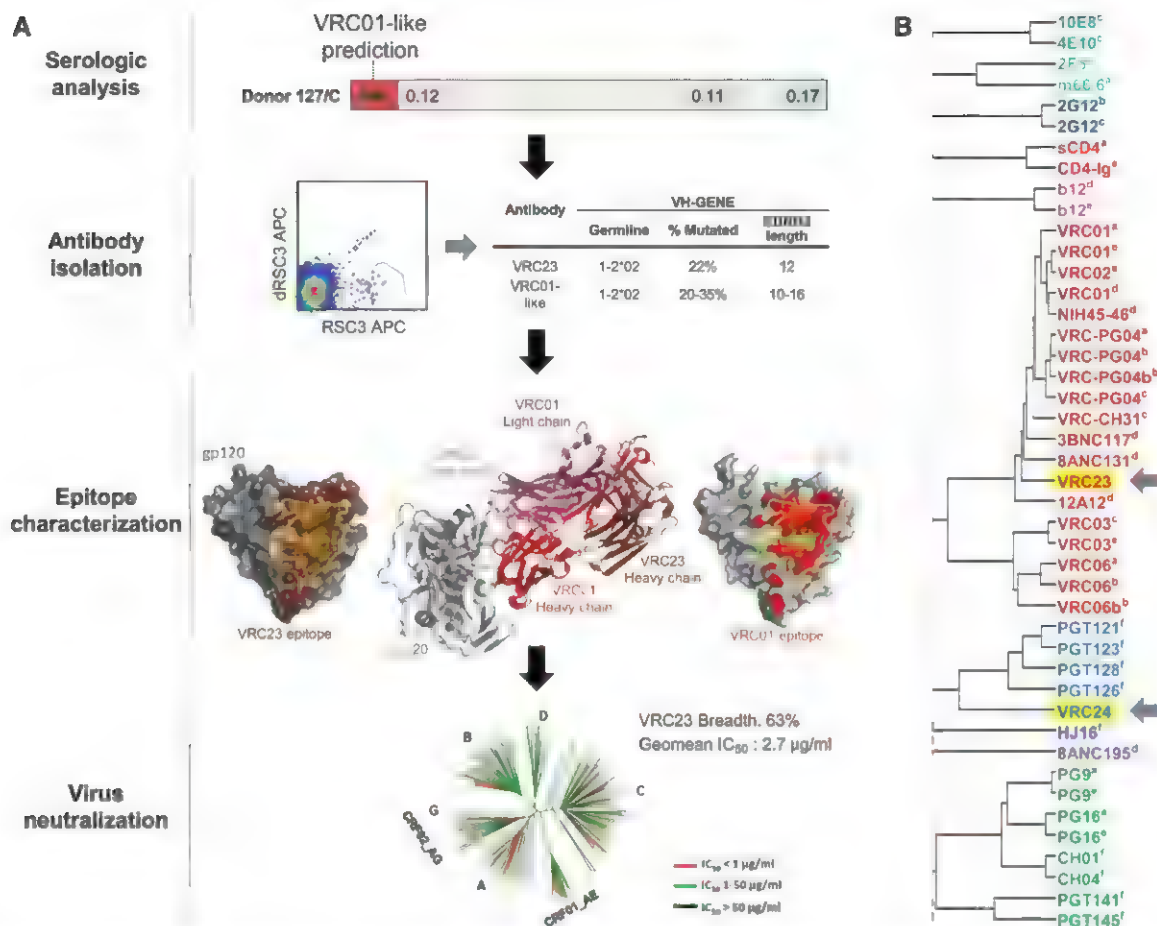


Fig. 4. Isolation and characterization of monoclonal antibodies isolated from sera identified by neutralization-based predictions. (A) Isolation and characterization of antibody VRC23 from donor 127/C. (Top row) Donor 127/C was predicted to have VRC01-like antibodies. (Row 2) Immunoglobulin G-positive (IgG+) B cells from donor 127/C were stained with the CD4bs-specific probe RSC3 and the CD4bs mutant dRSC3 and sorted. IgG variable genes were recovered by reverse transcription polymerase chain reaction, sequenced, and subcloned into heavy- and light-chain expression vectors. Like VRC01, the VRC23 heavy chain derives from the VH1-2*02 germline gene. The percent mutation from the germline VH gene is shown, as is the amino acid length (from the Kabat database) of the CDRH3 loop. (Row 3) VRC23-gp120 crystal structure shows gp120 recognition similar to that of VRC01. Polypeptide chains are depicted in ribbon representation

(middle). The footprints of VRC23 (left, brown) and VRC01 (right, red) on the surface of gp120 showed similar targeting of the CD4-defined initial site of vulnerability (yellow trace). (Bottom row) VRC23-neutralization dendrogram. VRC23 was tested against 178 genetically diverse Env-pseudoviruses representing the major HIV-1 clades. Neighbor-joining tree displays the protein distance of the respective gp160 sequences. Tree branches are colored by the neutralization potency of VRC23 against each particular isolate. The geometric mean IC_{50} of all neutralized viruses is shown. (B) Neutralization-based antibody clustering shows VRC23 (highlighted) clustering with the VRC01-like antibodies and VRC24 (highlighted) clustering with the glycan-dependent PGT128-like antibodies, in agreement with epitope determination by atomic-level structure (VRC23) and mapping by mutagenesis (VRC23 and VRC24).

currently identified sites are likely to dominate the neutralizing antibody response, highly effective antibodies against unknown and/or less-well-characterized epitopes continue to be identified (53, 62). Generally, epitope delineation based on neutralization fingerprints may provide a transformative strategy for screening sera or characterizing antibody specificities induced upon infection or vaccination against HIV-1 as well as other viruses.

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42. One of the features of our approach is its ability to distinguish between antibodies targeting overlapping epitopes in a substantially different ways: There is a significant difference in the correlation coefficients for antibodies targeting a similar epitope versus the correlation coefficients for antibodies targeting different epitopes on the same site of vulnerability. Similarly, there is a significant difference for similar epitopes versus different sites of vulnerability; however, there is no significant difference for different epitopes on the same site of vulnerability versus different sites of vulnerability (fig. S1).
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48. The division into monoclonal antibody clusters was based on the clustering results from the larger 34-strain panel (fig. 2A), whereas serum delineation was performed on an available 21-strain panel (because of serum volume constraints, we used a 21-strain subset of the main panel). Although antibody clustering is sensitive to the size of the viral panel (appendix S1), predictions with smaller panels (such as the 21-strain panel used in the serum analysis) could nonetheless be sufficient (fig. S3); delineation of component-antibody specificities for the CAP256 sera was based on a smaller 13-strain panel (41).
49. A signal for PGT128-like antibodies in donor 45 was also observed. However, only part of the neutralization activity of the donor 45 serum could be attributed to VRC01-like antibodies (fig. S11), which indicated that other antibody specificities may also exist in that serum. It is thus not surprising to observe other neutralization signals in the predictions.
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54. For example, standard serum mapping for glycan-V3 antibodies uses N332 mutants and, thus, may not be as sensitive for some antibodies in the PGT128-like group that are more affected by glycan-301 (table S4).
55. The neutralization-based delineation showed relatively strong signals (>0.2) for 2G12- or b12-like antibodies with many of the sera, and potential VRC01-, PG9-, PGT128-, and 10E8-like specificities were predicted for specific subsets of the sera. In the case of 2G12-like activity, many serum samples were also found to be positive by experimental mapping assays, which indicated that this epitope might be a more frequent

target compared with other major epitopes, although not necessarily by antibodies having the specific domain-exchanged nature of 2G12 (fig. 3D). A clear threshold for the presence or absence of a particular specificity in a given serum was not apparent: A level of 0.35 positively identified 10E8-like antibodies in donor N152, but sporadic signals of up to ~0.3 were observed in longitudinal samples (fig. 3B).

56. To confirm the predicted similarity to VRC01, we crystallized and determined the structure of the antigen-binding fragment (Fab) of VRC23 in complex with an HIV-1 core gp120. The epitope footprints on gp120 for VRC23 and VRC01 were similar, with buried gp120 surface areas correlating significantly (Spearman $R = 0.76$, $P < 0.0001$) (fig. 4A and figs. S8 to S10).
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Supplementary Materials

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Materials and Methods
Supplementary Text
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Emergence of Individuality in Genetically Identical Mice

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Brain plasticity as a neurobiological reflection of individuality is difficult to capture in animal models. Inspired by behavioral-genetic investigations of human monozygotic twins reared together, we obtained dense longitudinal activity data on 40 inbred mice living in one large enriched environment. The exploratory activity of the mice diverged over time, resulting in increasing individual differences with advancing age. Individual differences in cumulative roaming entropy, indicating the active coverage of territory, correlated positively with individual differences in adult hippocampal neurogenesis. Our results show that factors unfolding or emerging during development contribute to individual differences in structural brain plasticity and behavior. The paradigm introduced here serves as an animal model for identifying mechanisms of plasticity underlying nonshared environmental contributions to individual differences in behavior.

Plasticity, or the reciprocal interaction between brain structure and function, draws on genetic and nongenetic sources of variation and forms the neurobiological basis of individuality. Behavioral-genetic studies with humans provide statistical tools for estimating the additive and interactive contributions of genetic and environmental variations to individual differences in behavioral development (1). In the case of monozygotic twins reared together, sibling differences reflect the influence of individual responses, based on the same genetic makeup, to a nominally identical environment. Somewhat paradoxically, this source of variation is generally

referred to as the "nonshared environment" in behavior genetics. As Turkheimer has noted, "exactly what the nonshared environment consists of has been a matter of mystery and controversy for some time" [(2) p. 826]. We developed an animal model for studying the nonshared environment and examined its effects on behavioral and neural development.

In rodents, enriched environments are among the tools of choice for addressing the influence of a given environment on individuals with identical genetic background (3, 4). However, with some exceptions [(5), see also (3, 6)] the emergence of experience-based individual differences within groups of genetically identical animals exposed

to the same enriched environment has rarely been addressed. We used a large group of animals and a particularly complex environment to capture the emergence of individual differences in brain and behavior over time. We used exploration as a marker of behavioral development, and adult neurogenesis in the hippocampus as a marker for continued brain development.

Adult hippocampal neurogenesis allows life-long plastic adaptation of the hippocampal neural network in the face of environmental complexity and novelty, is regulated by physical and cognitive aspects of behavioral activity, and can be quantified in a straightforward way and captured with numerical key parameters (7). We used the individual regulation of adult hippocampal neurogenesis in response to individual differences in experiencing a nominally identical environment as a proxy for individual brain plasticity. What happens when genetically identical mice inhabit the same environment? How much individuality emerges over time, and is it related to adult hippocampal neurogenesis? Do initial individual differences in behavior dominate later individual differences, or will novel variance emerge?

Forty female inbred mice (C57BL/6N), 4 weeks old at the beginning of the experiments, were kept in a large enriched environment (ENR) for

3 months [(8); see Fig. 1A and detailed description in the supplementary materials]. The mice were tagged with radio-frequency identification (RFID) transponders, and 20 antennas, distributed over the entire environment, monitored their current locations.

Over the experimental period of 3 months (Fig. 1B), we saw an increase in mean body weight (Fig. 1C) compared with the baseline group ($N = 8$). Besides this age effect, variability of both body and brain weight (Fig. 1D) seemed larger in ENR mice than in control (CTR) mice at the end of the experiment (but $P = 0.057$ for brain-weight variance, Bartlett-box F test, $F_{11,39} = 0.338$; and $P = 0.154$ for body-weight variance, $F_{11,39} = 0.449$).

Adult neurogenesis was assessed at the end of the experiment by counting proliferating precursor cells that had been labeled with bromodeoxyuridine (BrdU) 3 weeks before (Fig. 1B). Average adult neurogenesis was increased in the ENR group compared with the CTR group (Fig. 2A), decreasing the physiological age-related decline in adult neurogenesis (as shown in the comparison with the baseline value at 8 weeks). In line with previous studies, ENR animals also had significantly more new astrocytes than CTR animals ($t = 4.321$, $P = 0.0002$) but there was no significant difference in cells with undefined phenotype ($t = 0.736$, $P = 0.5$) (fig. S1) (9).

New neurons in the hippocampus are assumed to enable the hippocampus to flexibly cope with novelty and complexity (10). Physical activity and locomotion provide subjective proxy feedback signals that tend to indicate situations potentially rich in cognitive challenges requiring plasticity (11, 12). Our specific hypothesis was that mice with a greater range of experiences, as reflected in more explorative behavior corresponding to a larger and more intensive coverage of the territory, would show higher levels of adult hippocampal neurogenesis. Within the ENR group, individual differences in the total number of antenna contacts as proxy for the sheer amount of locomotion were not associated with individual

differences in neurogenesis [correlation coefficient (r) = 0.133; $t = 0.829$; $P = 0.412$] (fig. S2) (13). Hence, we sought to identify a trait marker of activity that would be more likely to be associated with adult neurogenesis.

To obtain an ethologically valid index of explorative behavior, we derived a new measure, called “roaming entropy” (RE; for more details, see methods in the supplementary materials). RE is the entropy of the probability distribution of finding a mouse at a given antenna at a given time and, thus, is an indicator of the territorial range covered by a given mouse in a given period of time. RE is a continuous measure. RE is low when a mouse has a stable and small home range, independent of the amount of locomotion within that area. But even a relatively large range can be covered with low RE if few stable spots of attendance are spread out over larger distances. RE is high, in contrast, if coverage is evenly distributed over the entire area of the cage (Fig. 2B; see also movies S1 and S2).

Measurements of RE were aggregated into four adjacent time periods (T1, T2, T3, and T4), each representing the average RE over 24 calendar days, and summed over time periods to obtain an index of cumulative roaming entropy (cRE; i.e., $cRE_{T1} = RE_{T1}$; $cRE_{T2} = cRE_{T1} + RE_{T2}$; $cRE_{T3} = cRE_{T2} + RE_{T3}$; $cRE_{T4} = cRE_{T3} + RE_{T4}$). A three-factor latent-growth curve model was fit to the data to obtain estimates of intercept, linear change, and exponential change (for details, see methods in the supplementary materials). The fit of the model was acceptable, comparative fit index (CFI) = 0.986, root mean square error of approximation (RMSEA) = 0.099. Although reliable individual differences in cRE were present at baseline, these differences were completely wiped out by individual differences that emerged during the observation period (Fig. 2C). The correlation between cRE and neurons labeled with BrdU and neuronal marker NeuN did not differ significantly from zero at baseline, $r = 0.24$ ($P = 0.144$). In contrast, at the end of the experiment,

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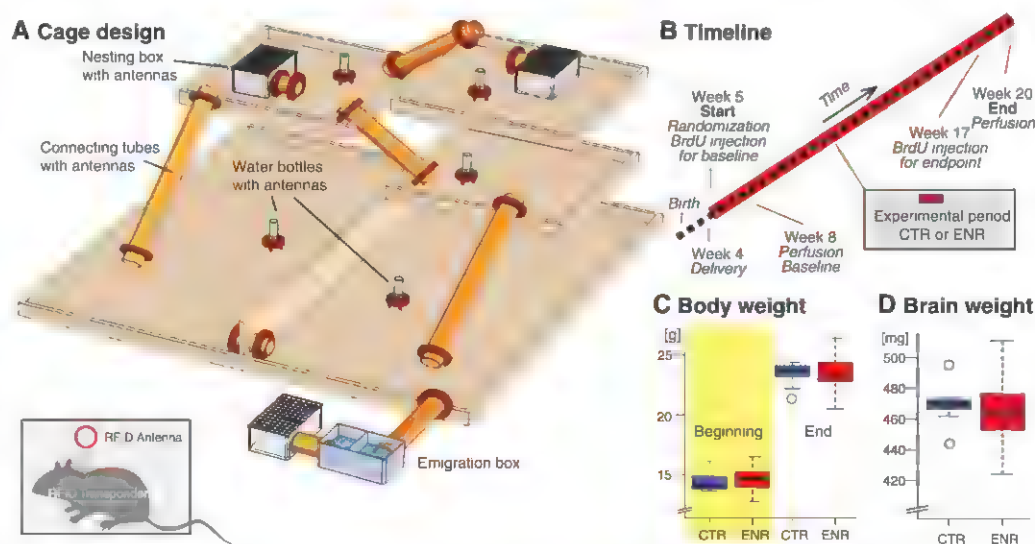
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Fig. 1. Experimental setup and effects on body and brain weight.

(A) Schematic illustration of the large enrichment enclosure housing 40 mice including RFID antenna positions (shown as red rings). Positions of levels, water sources, nesting boxes, and connecting tubes are drawn to scale. (Inset) Schematic illustration of animal tracking; an RFID passive integrated transponder (PIT) is implanted in mouse's neck. The electromagnetic field issued by the antenna induces the PIT to emit the number identifying the animal. This information is then picked up by the antenna and stored into a database together with spatial and temporal annotations. (B) Experimental time line. (C) Body weight development: weights (in grams) of CTR (blue) and ENR (red) mice at the beginning and end of the experiment. (D) Brain weights at perfusion (in grams). The difference in variance between CTR and ENR missed conventional statistical significance at $P = 0.057$.



the number of new neurons (BrdU and NeuN double-positive cells) correlated significantly with cRE at T4, $r = 0.46$ ($t = 3.227$, $P = 0.0026$). Mice who explored their habitat more broadly also grew more new neurons in the hippocampus (Fig. 2D). An estimate of distance traveled (i.e., number of unique, nonrepetitive antenna contacts over the period of the experiment) showed a weaker but still significant association with adult neurogenesis ($r = 0.345$; $t = 2.268$; $P = 0.029$), which explained 12% of the variance (fig. S2 and related information).

This study shows that adult neurogenesis, as an instantiation of brain plasticity, is linked to individual differences in experience among genetically identical individuals who live in a nominally identical environment. About one-fifth of the experiential effects on adult neurogenesis was captured by a measure of roaming through an enriched environment. This finding supports the idea that the key function of adult neurogenesis is to shape hippocampal connectivity according to individual needs and thereby to improve adaptability over the life course and to provide evolutionary advantage (11, 14, 15). The observed individual differences in behavioral trajectories were reliable. This is in line with the observation that behavioral traits can be strongly influenced by external stimuli that vary between individuals or populations of individuals, as evidenced by diverging results of behavioral testing across different laboratories (16, 17).

The molecular mechanisms driving individual differences in behavioral and neural plasticity await further study. From the present results, three routes seem worth pursuing: (i) As full inbreeding is impossible, minimal residual segregation remains, and ontogeny may amplify the functional consequences of this residual genetic variation. However, this variability due to novel mutations corresponds to only 8 to 12 single-nucleotide polymorphisms across the entire genome (18). In addition, inbred mice might possibly genetically vary with respect to variable number tandem repeats and transposon insertions (19, 20) (ii) Stochastic gene regulation may lead to individual differences in molecular states, which are further amplified through experience. (iii) Animals might show small changes in the epigenetic state of their genome and may drift epigenetically apart over time, which reflects the cumulative effects of the choices they make in the course of their lives. This last explanation would be particularly in line with Turkheimer's argument (2) and consistent with data from human monozygotic twins, according to which epigenetic differences increase from young adulthood to old age and contribute to a growing discordance of monozygotic twins with advancing age (21). In addition, Lathe has suggested the following sources of initial individuality in rodents (19): intrauterine position, nutrition and interaction, imprinting errors, maternal stress and disease, and early postnatal interactions (including handling). Presumably, all of these would result in differences in the epigenome. When we obtained the animals for

our study, we received mice randomly picked from as many litters as possible to achieve the best possible randomization.

As foreshadowed by psychological (22) and neurobiological (23) theories of ontogenetic development and in line with general theories of neural self-organization (24), small perturbations,

possibly related to the factors mentioned above, may lead to initial individual differences in action tendencies. These differences, in turn, may trigger differences in experience that accumulate over time, result in differential plasticity, and correspond to different epigenetic states and developmental trajectories.

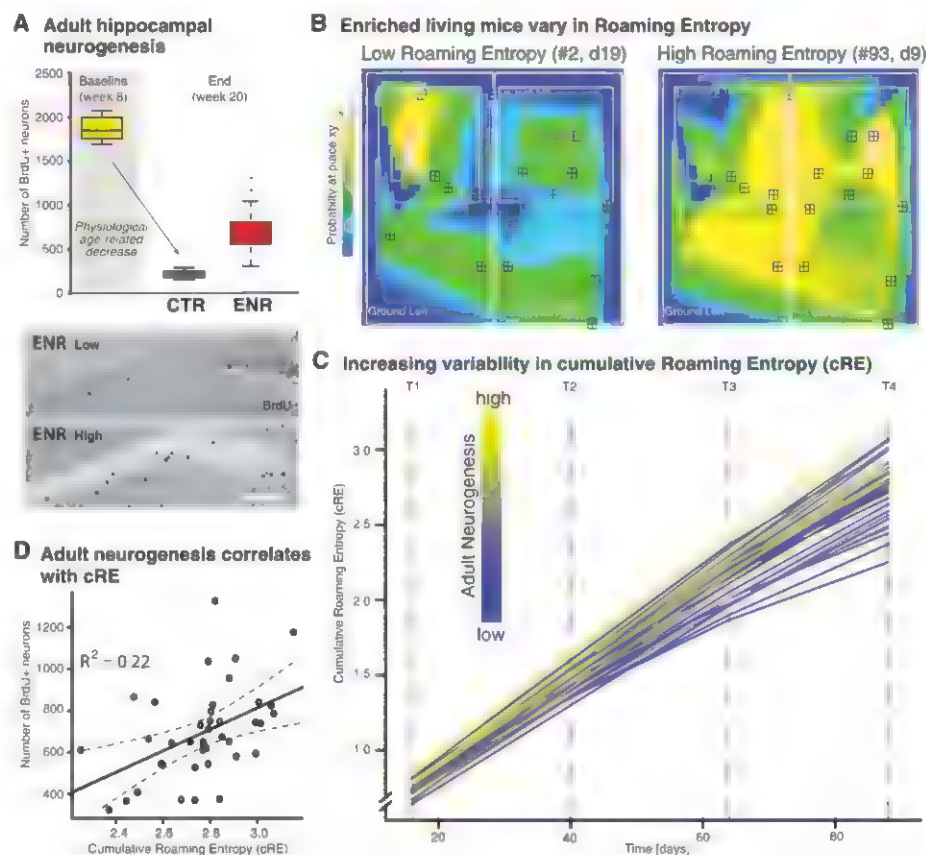


Fig. 2. Adult hippocampal neurogenesis and RE. (A) Total number of new neurons (BrdU+ and NeuN+ cells) in the hippocampal dentate gyrus. Both CTR and ENR groups show the typical age-related decrease in neurogenesis. Compared with CTR mice, ENR mice increased adult hippocampal neurogenesis and resulted in a relative increase in variability. (Baseline versus CTR: $P = 5.82 \times 10^{-9}$; baseline versus ENR: $P = 7.17 \times 10^{-12}$, CTR versus ENR: $P = 7.68 \times 10^{-5}$.) Exemplary histological images. (Top) "ENR low" shows the dentate gyrus of an animal with an adult neurogenesis level in the range of CTR, whereas the mouse in "ENR high" is at the upper end of neurogenesis levels in ENR. Scale bar, 150 μ m. All analyses were done on continuous data, not by classifying animals into categories of high or low neurogenesis. (B) Exemplary heat maps of the RE of two mice in the large ENR enclosure (see also supplementary materials). The two panels are heat maps of explorative behavior for two mice assessed during one night depicting the probability of a mouse being in a specific location when viewed from above (i.e., aggregating across the four levels of the cage). Low probabilities are shown in blue, medium probabilities in green, and high probabilities in orange (see arrow on the left). Antenna positions are shown in black. (Left) A mouse with low RE (animal no. 2 at day 19); (right) a mouse with high RE (animal no. 93 at day 9). All analyses were done on continuous data, not by classifying animals into categories of high or low RE. (C) Measurements of RE were aggregated into four adjacent time periods to obtain an index of cumulative RE (cRE). Each line displays the cRE for a single mouse. Corresponding levels of neurogenesis are continuously color-coded from low (blue) to high (yellow). For exact values, see also the scatterplots in Fig. 2D. The mice differed in cRE at T1 (variance T1 = 0.001, $\chi^2 = 28.91$, $df = 1$, $P < 0.0001$). At the same time, they differed markedly in rates of linear change ($\chi^2 = 18.76$, $df = 1$, $P < 0.0001$) and exponential change ($\chi^2 = 27.80$, $df = 1$, $P < 0.0001$). As a result, the variance in cRE at T4 (variance T4 = 0.022, $\chi^2 = 35.12$) increased by a factor of 22 relative to the variance in cRE at T1 (for the difference, $\chi^2 = 31.73$, $df = 1$, $P < 0.0001$). Individual differences in linear change predicted individual differences in cRE at the end of the observation period, $r = 0.98$ ($\chi^2 = 118.742$, $P < 0.0001$), whereas individual differences in cRE at the beginning of the observation period did not predict individual differences in linear change ($\chi^2 = 3.19$, $P = 0.074$). (D) Individual differences in cRE are associated with individual differences in adult hippocampal neurogenesis. The number of new neurons correlated significantly with cRE at T4, $r = 0.46$ ($t = 3.227$, $P = 0.0026$).

Environmental enrichment does not seem generally to increase variability, although some controversy exists with regard to parameters such as body weight (17, 25, 26). Most studies, however, have followed smaller cohorts of animals over shorter periods of time than in our study. Whether long-term enrichment in large groups and seminaturalistic conditions have a general variance-increasing effect across a wide range of parameters remains to be determined.

Three months of living in a complex environment led to a massive magnification of individual differences in explorative behavior among genetically identical individuals over time, and these differences were related to adult hippocampal neurogenesis. The rich environment lost its "sameness" over time and gave way to the emergence of a personalized "life space" (27) and a "mouse individuality," similar to what has been observed in humans for personality traits (28). Hence, the magnitude of individual differences observed in replications of this experiment is likely to vary across studies: As the members of each new cohort individualize, their "society" will also be shaped in a slightly different, individual way. The present paradigm serves as an animal model for addressing the "mystery and controversy" (2) of

the nonshared environment, or the ways in which living our lives makes us who we are (29).

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Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6133/756/DC1
Materials and Methods
Figs. S1 and S2
References (30–35)
Movies S1 and S2

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Compartmentalization of GABAergic Inhibition by Dendritic Spines

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γ -aminobutyric acid–mediated (GABAergic) inhibition plays a critical role in shaping neuronal activity in the neocortex. Numerous experimental investigations have examined perisomatic inhibitory synapses, which control action potential output from pyramidal neurons. However, most inhibitory synapses in the neocortex are formed onto pyramidal cell dendrites, where theoretical studies suggest they may focally regulate cellular activity. The precision of GABAergic control over dendritic electrical and biochemical signaling is unknown. By using cell type-specific optical stimulation in combination with two-photon calcium (Ca^{2+}) imaging, we show that somatostatin-expressing interneurons exert compartmentalized control over postsynaptic Ca^{2+} signals within individual dendritic spines. This highly focal inhibitory action is mediated by a subset of GABAergic synapses that directly target spine heads. GABAergic inhibition thus participates in localized control of dendritic electrical and biochemical signaling.

A challenge to elucidating the function of synaptic inhibition is the diversity of γ -aminobutyric acid–releasing (GABAergic) interneurons found in cortical circuits (1–3). Several interneuron classes, including those that express somatostatin (SOM-INs), target the dendrites of excitatory, glutamatergic pyramidal cells (3–5). SOM-INs regulate the initiation of action potential bursts generated via active currents in postsynaptic dendrites (6–8). We hypothesized that these inputs might also exert focal influence over dendritic signaling. Here, we used electrophysiological, optical, and computational approaches to investigate the localized actions of GABAergic inhibition in pyramidal cell dendrites.

To activate dendritic GABAergic synapses, we used a somatostatin-Cre mouse line (9) (Fig. 1A

and fig. S1, A and B) to conditionally express channelrhodopsin-2 (ChR2) (10) in SOM-INs of the prefrontal cortex (fig. S1, C and D). In acute brain slices prepared 2 to 3 weeks after viral injection, pulses of light (5 ms, 473 nm) delivered through the microscope objective evoked action potentials (APs) in fluorescently identified SOM-INs (fig. S2, A to C). Whole-cell recordings in layer 2/3 pyramidal neurons revealed corresponding inhibitory postsynaptic potentials (IPSPs) (Fig. 1, B and C, and fig. S2, D to F). For subsequent experiments, type A GABA receptor (GABA_A)–mediated IPSPs were isolated by including the selective type B GABA receptor (GABA_B) antagonist CGP-55845 in the perfusate (Fig. 1C). IPSPs exhibited a reversal potential of -69.9 ± 1.5 mV (mean $\pm$ SEM, $n = 5$)

that did not differ significantly from the value recorded via gramicidin-based perforated patch (-72.4 ± 1.7 , $n = 6$, $P = 0.3$, fig. S2, G and H).

To determine how inhibition influences dendritic activity in pyramidal neurons, we used two-photon laser scanning microscopy (2PLSM) to image calcium (Ca^{2+}) in apical dendritic spines and shafts. Ca^{2+} transients (ΔCa^{2+}) were evoked by somatic APs (Fig. 1, D and E, and fig. S3, A and B) and were mediated by voltage-gated Ca^{2+} channels (VGCCs) (fig. S3C). We compared AP-evoked Ca^{2+} signals under control conditions ($\Delta\text{Ca}^{2+}_{\text{ctrl}}$) and when preceded by an IPSP ($\Delta\text{Ca}^{2+}_{\text{inh}}$) (15-ms interval) evoked by a light pulse targeting the imaged region (Fig. 1E). In 57% (73/127) of randomly imaged spines, optical activation of SOM-INs produced a significant reduction ($>15\%$, see methods and fig. S3, D to F) in the AP-evoked ΔCa^{2+} . At these locations, the average Ca^{2+} inhibition ($\Delta\text{Ca}^{2+}_{\text{inh}}/\Delta\text{Ca}^{2+}_{\text{ctrl}}$) was significantly greater for spines than for neighboring dendritic shafts (0.60 ± 0.02 versus 0.78 ± 0.03 , $P < 0.001$; Fig. 1, F and G). The inhibition of ΔCa^{2+} was abolished by application of the GABA_A antagonist picrotoxin ($n = 8$, $P < 0.05$, Fig. 1H). Similar Ca^{2+} inhibition was seen in basal dendrites (23/49 spines; 0.73 ± 0.02 versus 0.87 ± 0.02 for spines and shafts, respectively, $P < 0.01$; fig. S4).

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We frequently observed inhibited and uninhibited spines in close proximity, suggesting compartmentalized GABAergic control of Ca^{2+} signaling. We therefore imaged Ca^{2+} inhibition within a small dendritic region. Spines adjacent to an inhibited reference spine typically showed little modulation despite the presence of a somatic IPSP (Fig. 2, A and B, and fig. S5, A and B). We generated “maps” demonstrating heterogeneous inhibition over short distances (Fig. 2C). There was significantly greater inhibition for each reference spine than for its adjacent neighbor (0.58 ± 0.03 versus 0.82 ± 0.03 , $P < 0.001$, $n = 22$ maps), and inhibition between neighbors was not correlated (Pearson $r^2 = 0.12$, $P = 0.09$, Fig. 2H). Inhibition in individual spines was not correlated to the magnitude of $\Delta\text{Ca}^{2+}_{\text{ctl}}$ (fig. S5C) and was unchanged for experiments conducted at near-physiological temperature or with GABA_AR function intact (fig. S5D).

We further characterized inhibitory compartmentalization by using photoactivation of the caged compound RuBi-GABA (11). Brief light pulses (2 ms, 473 nm) evoked IPSPs in pyramidal neurons bathed in RuBi-GABA (10.8 μM) with much smaller amplitudes but similar kinetics as those produced by stimulation of SOM-INs (fig. S6, A to C). By using GABA uncaging, we found that 51% (44/87) of randomly imaged apical spines showed significant inhibition of ΔCa^{2+} . Inhibition was stronger in spines than in dendritic shafts (0.65 ± 0.02 versus 0.79 ± 0.03 , $P < 0.0001$, $n = 59$; Fig. 2, G and fig. S6D) and was blocked by picrotoxin (fig. S6, E and F). Inhibitory compartmentalization was similar to

that seen by using optical stimulation of SOM-INs (Fig. 2, D to F). Ca^{2+} inhibition for the reference spine was significantly greater than for the adjacent neighbor (0.64 ± 0.05 versus 0.90 ± 0.06 , $P < 0.01$, $n = 15$ maps), and these values were uncorrelated (Pearson $r^2 = 0.11$, $P = 0.22$, Fig. 2I).

Increasing intracellular chloride caused IPSPs to be depolarizing from a membrane potential (V_m) of -60 mV and largely eliminated inhibition of ΔCa^{2+} ($n = 24$, $P < 0.0001$ versus control, fig. S7, A and B), suggesting that local membrane hyperpolarization contributes to reduced Ca^{2+} influx. VGCCs with more depolarized activation thresholds should therefore exhibit greater sensitivity to GABAergic inhibition. Indeed, blockade of high-threshold (L and N/P/Q types) but not lower-threshold (T and R types) channels significantly reduced the amount of Ca^{2+} inhibition evoked by GABA uncaging (fig. S7C).

Many spines receive direct GABAergic input (5, 12, 13), and we wondered whether SOM-INs might contribute to this pool of synapses. We reconstructed apical dendrites of recorded neurons from Chr2 experiments and found 18.5% of spines ($n = 1185$ spines, 4 cells) expressed the inhibitory synaptic protein gephyrin, whereas 43.5% ($n = 3058$ spines, 9 cells) appeared to be contacted by a presynaptic bouton originating from a SOM-IN (fig. S8, A and B; see methods). For a subset of cells ($n = 3$), we recovered spines with corresponding Ca^{2+} imaging. In all cases, Ca^{2+} inhibition was only observed for spines with an apposed SOM-IN terminal (Fig. 3, A to C, and fig. S8, C to H).

We next confirmed the spatial precision of GABAergic inhibition by using diffraction-

limited two-photon laser uncaging (2PLU) of 4-carboxymethoxy-5,7-dinitro-indolyl (CDNI)-GABA (14) (methods). The Ca^{2+} inhibition evoked by 2PLU_{GABA} was similar in magnitude to that produced by Chr2 activation, highly sensitive to the precise location of the uncaging spot around the spine perimeter, and isolated from neighboring spines (Fig. 3, D to G).

To see whether GABAergic synapses onto spines are necessary for compartmentalized inhibition, we simulated AP-evoked Ca^{2+} influx into dendritic spines and shafts (Fig. 3H, fig. S9, and methods). GABAergic input to a single spine head inhibited ΔCa^{2+} only in the targeted spine, whereas inhibition targeting the dendritic shaft had minimal effect on nearby spines (Fig. 3I). Moreover, ΔCa^{2+} in the dendritic shaft was unaffected by GABAergic input to either the spine head or shaft (Fig. 3J). Ca^{2+} inhibition was mediated by a compartmentalized reduction in input impedance, reducing AP amplitude in the targeted spine (fig. S9, A to D). The magnitude of inhibition was influenced by spine neck resistance, chloride reversal potential, and VGCC activation threshold but was independent of VGCC density (fig. S9, E to G). Inhibition ($>15\%$) of dendritic ΔCa^{2+} could only be obtained by increasing the dendritic GABAergic conductance 10-fold (fig. S9H). A current-based inhibitory synapse that generated a similar IPSP in the absence of a conductance change produced minimal Ca^{2+} inhibition (fig. S9I).

Lastly, we asked whether localized inhibition occurred for synaptic Ca^{2+} transients and excitatory postsynaptic potentials (EPSPs). We combined one-photon RuBi-GABA uncaging with

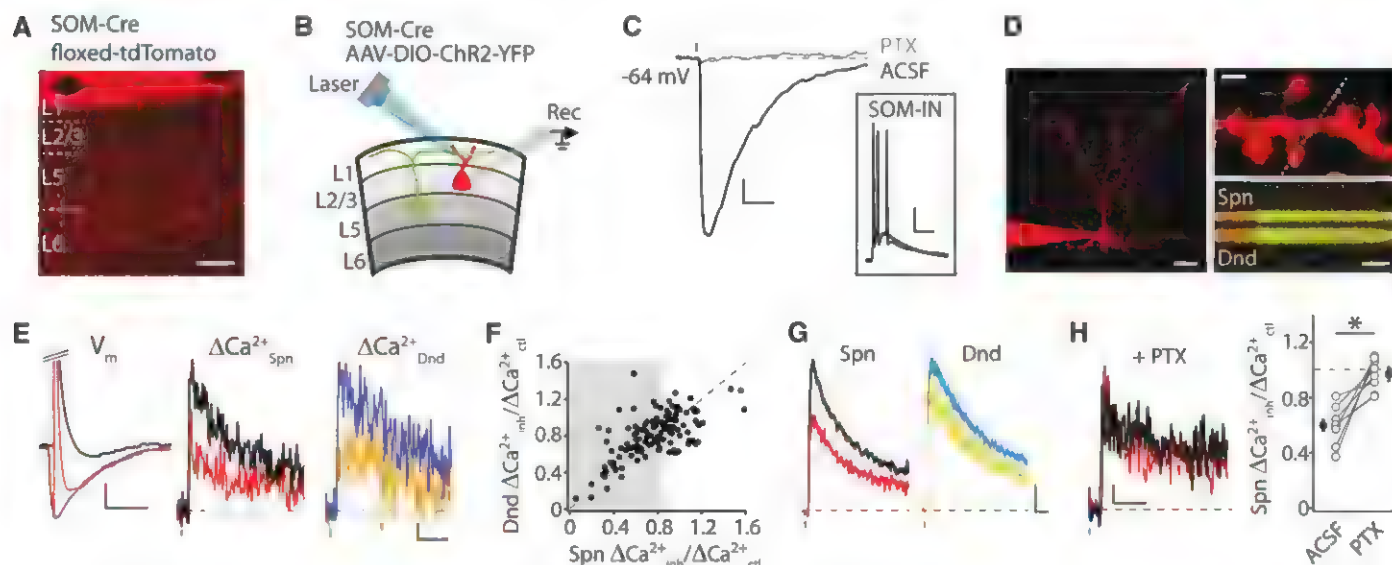


Fig. 1. SOM-INs mediate inhibition of dendritic Ca^{2+} signals. (A) tdTomato expression in the prefrontal cortex of SOM-Cre;Ai9 mice. Scale bar indicates 200 μm . (B) Recording configuration. (C) Light-evoked IPSPs (ACSF) are abolished by picrotoxin (PTX). Scale bars, 1 mV, 50 ms. (Inset) Light-evoked APs in a SOM-IN. Scale bars, 20 mV, 50 ms. (D) (Left) 2PLSM image of a layer 2/3 pyramidal neuron. Scale bar, 25 μm . (Right) AP-evoked ΔCa^{2+} in the spine (Spn) and dendritic shaft (Dnd) indicated by the dashed line. Scale bars, 1 μm , 50 ms. (E) (Left) V_m during AP (black), IPSP (blue), and IPSP-AP (red). Scale bars, 2 mV,

100 ms. (Middle and right) ΔCa^{2+} (Spn, Dnd) in response to AP (black, blue) or IPSP-AP (red, orange). Scale bars, 1% $\Delta G/G_{\text{sat}}$ (where G indicates the amount of green fluorescence), 100 ms. (F) Ca^{2+} inhibition for dendritic shafts versus spines. Gray region indicates significant spine inhibition. (G) Average Ca^{2+} signals ($\pm$ SEM) evoked by AP or IPSP-AP for locations showing significant inhibition. Scale bars, 1% $\Delta G/G_{\text{sat}}$, 50 ms. (H) (Left) Ca^{2+} transients from spine in (E), recorded in PTX. Scale bars, 1% $\Delta G/G_{\text{sat}}$, 100 ms. (Right) Average Ca^{2+} inhibition before ACSF and after GABA_A block (PTX). * $P < 0.05$ (paired Student's t test).

2PLU of CDNI-glutamate [2PLU_{Glu} (15) (methods)] and imaged ΔCa^{2+} in spines (Fig. 4, A to D). Inhibition of synaptic ΔCa^{2+} was strongly compartmentalized with no correlation between neighboring spines (0.60 ± 0.05 versus 0.98 ± 0.05 for reference spine and adjacent neighbor, respectively, $P < 0.01$; $n = 12$; Pearson $r^2 = 0.18$; $P = 0.17$; Fig. 4, A to D). The 2PLU_{Glu}-evoked

EPSPs were similarly inhibited (Fig. 4, B and D), exhibiting reductions in both amplitude and duration (Fig. 4, D and E) that suggested inhibition might influence synaptic integration. GABAergic input significantly reduced the summation of responses evoked by glutamate uncaging on neighboring spines ($P < 0.05$, $n = 11$, Fig. 4, F to H). The effect on summation was eliminated after block-

ing *N*-methyl-D-aspartate-type glutamate receptors (NMDARs, $n = 7$, Fig. 4, G and H). Furthermore, summation was not reduced for cases where local GABA_A receptor activation evoked an IPSP but did not inhibit spine ΔCa^{2+} ($n = 8$, fig. S10).

Our results indicate that dendritic spines compartmentalize GABAergic inhibition, limiting both AP- and synaptically evoked Ca^{2+} influx and

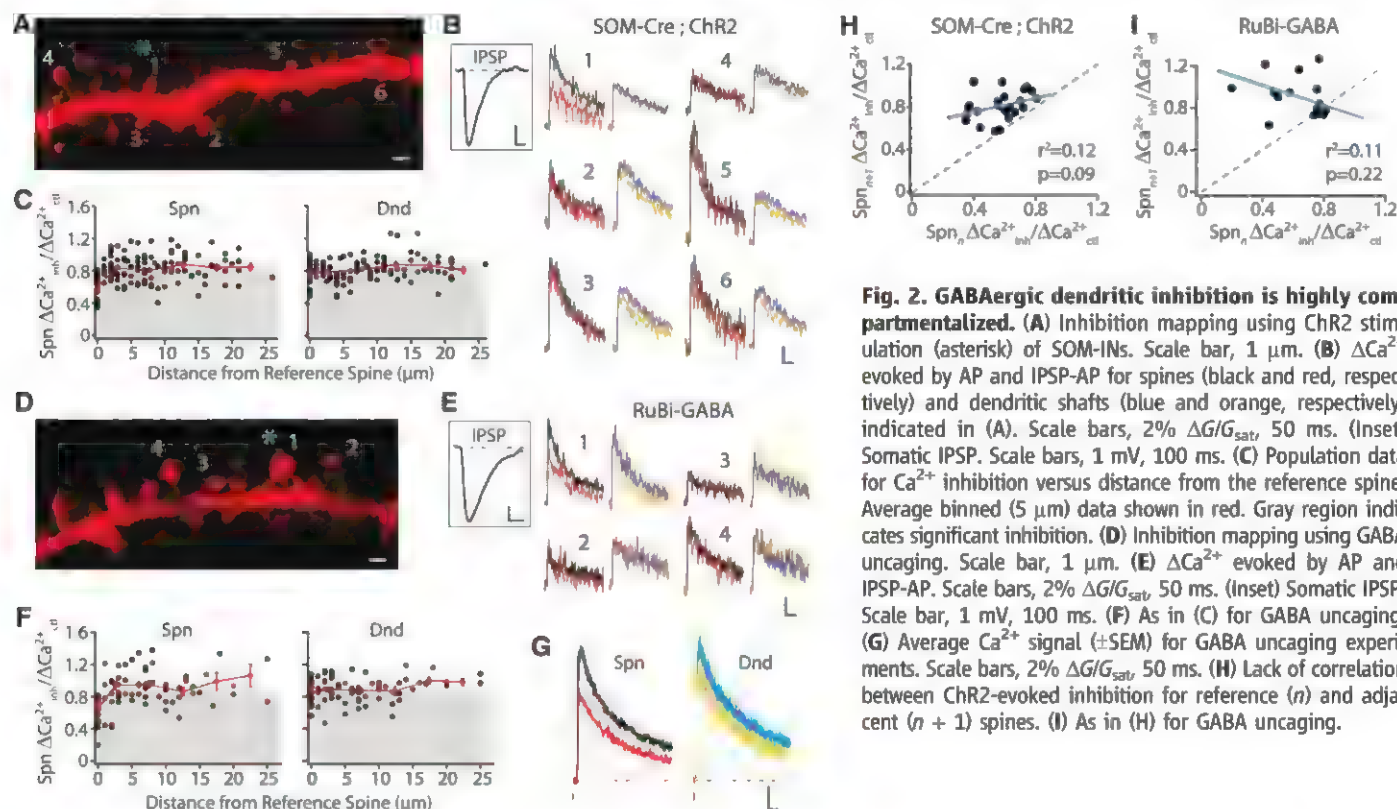


Fig. 2. GABAergic dendritic inhibition is highly compartmentalized. (A) Inhibition mapping using ChR2 stimulation (asterisk) of SOM-INs. Scale bar, 1 μm . (B) ΔCa^{2+} evoked by AP and IPSP-AP for spines (black and red, respectively) and dendritic shafts (blue and orange, respectively) indicated in (A). Scale bars, 2% $\Delta\text{G}/\text{G}_{\text{sat}}$, 50 ms. (Inset) Somatic IPSP. Scale bars, 1 mV, 100 ms. (C) Population data for Ca^{2+} inhibition versus distance from the reference spine. Average binned (5 μm) data shown in red. Gray region indicates significant inhibition. (D) Inhibition mapping using GABA uncaging. Scale bar, 1 μm . (E) ΔCa^{2+} evoked by AP and IPSP-AP. Scale bars, 2% $\Delta\text{G}/\text{G}_{\text{sat}}$, 50 ms. (Inset) Somatic IPSP. Scale bars, 1 mV, 100 ms. (F) As in (C) for GABA uncaging. (G) Average Ca^{2+} signal ($\pm\text{SEM}$) for GABA uncaging experiments. Scale bars, 2% $\Delta\text{G}/\text{G}_{\text{sat}}$, 50 ms. (H) Lack of correlation between ChR2-evoked inhibition for reference (n) and adjacent ($n + 1$) spines. (I) As in (H) for GABA uncaging.

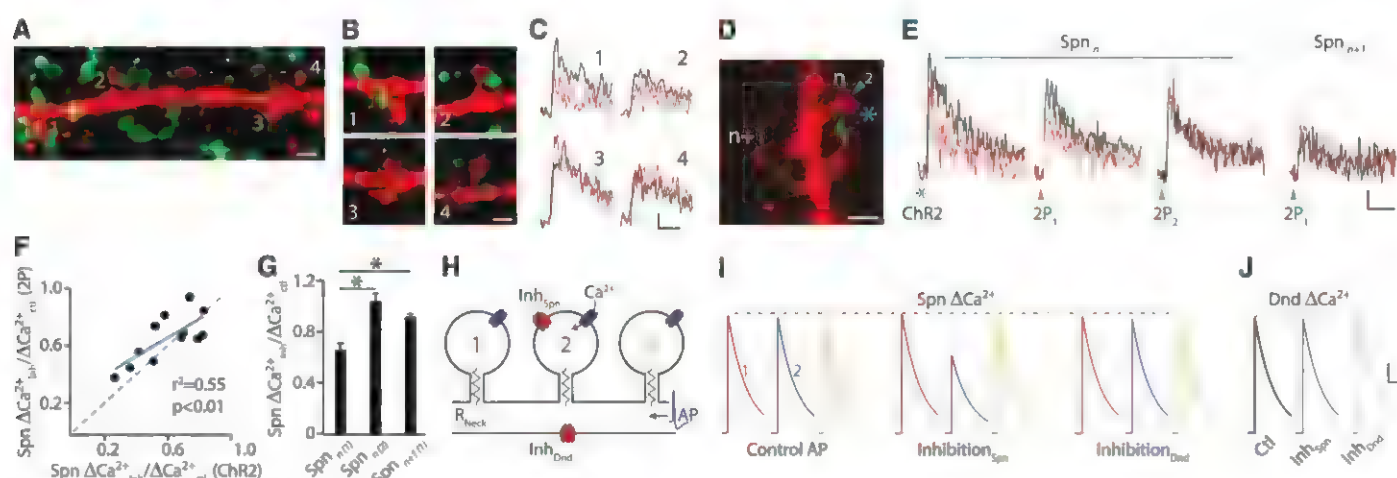


Fig. 3. GABAergic synapses on spines mediate local Ca^{2+} inhibition. (A) Confocal projection of a dendrite (red) and ChR2-enhanced yellow fluorescent protein-positive boutons (green). Scale bar, 1 μm . (B) Single-section images of spines from (A). Scale bar, 1 μm . (C) ΔCa^{2+} measured in spines from (B) for AP (black) and IPSP-AP (red). Scale bars, 1% $\Delta\text{G}/\text{G}_{\text{sat}}$, 50 ms. (D) Inhibition mapping using ChR2 (asterisk) or 2PLU_{GABA} (arrowheads). Scale bar, 1 μm . (E) ΔCa^{2+} in reference spine (n) and neighbor ($n + 1$) for AP (black)

and IPSP-AP (red). Scale bars, 2% $\Delta\text{G}/\text{G}_{\text{sat}}$, 50 ms. (F) Correlation between ChR2- and 2PLU_{GABA}-evoked inhibition. (G) Average Ca^{2+} inhibition ($\pm\text{SEM}$) evoked by 2PLU_{GABA}. (H) Computational model of dendritic inhibition. (I) Simulated GABAergic input selectively inhibits ΔCa^{2+} in spine 2. GABAergic input onto dendritic shaft has minimal effect on ΔCa^{2+} . (J) GABAergic input does not inhibit Ca^{2+} in the dendritic shaft. Scale bars in (I) and (J), 100 nM, 100 ms. * $P < 0.05$ (paired Student's t test).

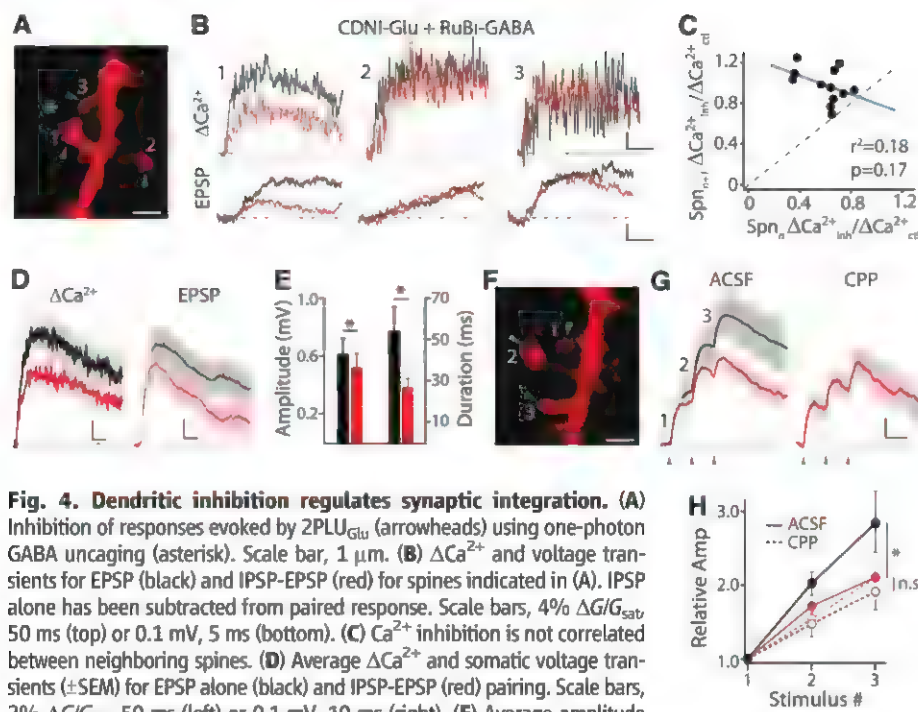


Fig. 4. Dendritic inhibition regulates synaptic integration. (A) Inhibition of responses evoked by 2PLU_{Glu} (arrowheads) using one-photon GABA uncaging (asterisk). Scale bar, $1\text{ }\mu\text{m}$. (B) ΔCa^{2+} and voltage transients for EPSP (black) and IPSP-EPSP (red) for spines indicated in (A). IPSP alone has been subtracted from paired response. Scale bars, $4\% \Delta\text{G}/\text{G}_{\text{sat}}$, 50 ms (top) or 0.1 mV , 5 ms (bottom). (C) Ca^{2+} inhibition is not correlated between neighboring spines. (D) Average ΔCa^{2+} and somatic voltage transients ($\pm\text{SEM}$) for EPSP alone (black) and IPSP-EPSP (red) pairing. Scale bars, $2\% \Delta\text{G}/\text{G}_{\text{sat}}$, 50 ms (left) or 0.1 mV , 10 ms (right). (E) Average amplitude and duration ($\pm\text{SEM}$) of voltage transients for EPSP (black) and IPSP-EPSP pairing (red). (F) Integration of responses evoked by 2PLU_{Glu} (arrowheads). Scale bar, $1\text{ }\mu\text{m}$. (G) Average voltage transients ($\pm\text{SEM}$) for EPSP (black) and IPSP-EPSP (red) evoked by 2PLU_{Glu} on three neighboring spines, recorded in control ACSF (left) or with NMDARs blocked by carboxypiperazin-4-yl-propyl-1-phosphonic acid (CPP) (right). Scale bars, 0.25 mV , 20 ms . (H) Relative summation of EPSPs (black) or IPSP-EPSPs (red), recorded in control ACSF or with CPP. * $P < 0.05$ (Wilcoxon matched pairs test); n.s., not significant.

regulating NMDAR-dependent synaptic integration. These findings establish a previously unknown mechanism for the synapse-specific control of Ca^{2+} signaling and downstream cellular processes such as synaptic plasticity.

Theoretical studies suggested that inhibition might regulate dendritic signaling near synaptic contacts [(16–18), but see (19)]. Subsequent experimental data demonstrated that GABA receptors can inhibit regenerative voltage-dependent dendritic spikes, controlling the production of AP bursts at the soma (6–8, 20). These findings were mediated in part by GABA_B -dependent modulation of VGCCs and NMDARs (8, 21) and suggested that inhibition acts with lower spatial resolution than glutamatergic excitation, which exhibits compartmentalization of electrical and biochemical signals within single spines (22, 23). However, our data indicate that the spine head similarly restricts GABA_A -mediated inhibition. The model further suggests that, in addition to the chloride reversal potential, spine neck resistance influences the efficacy of GABA_A synapses onto spine heads as occurs for glutamatergic inputs (24–26). Notably, our experimental data was closely modeled by using a neck resistance of 520 Mohm , similar to the value reported for hippocampal pyramidal neurons (26). Both neck resistance and chloride reversal are modulated by development and experience (27, 28), suggesting the impact of dendritic inhibition may be similarly regulated.

Dendritic Ca^{2+} influx plays a key role in the induction of plasticity at glutamatergic synapses (29), and inhibition can serve as a negative regulator of plasticity (30–32). Our results suggest that this control occurs at a previously unappreciated spatial scale, enabling dendrite-targeting interneurons to influence individual glutamatergic inputs. This observation is particularly relevant given the growing attention to links between perturbed GABA_A inhibition, alterations in developing neuronal circuits, and neuropsychiatric disorders such as schizophrenia and autism (33, 34).

Why do certain spines receive GABA_A inputs? One possibility is that GABA_A receptors are recruited by the presence of specific glutamatergic afferents, as proposed for thalamorecipient spines in frontal or visual cortex (35, 36). Additionally, recruitment of GABA_A receptors might be activity-dependent (37). This hypothesis is supported by evidence that spine-targeting GABA_A inputs exhibit distinctly high rates of turnover in vivo (12, 36). Future experiments are necessary to determine the existence of feedback loops between dendritic Ca^{2+} signals and the formation and stabilization of GABA_A synapses.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6133/759/DC1
Materials and Methods
Figs. S1 to S10
References (38–42)

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Microbiomics: The Germ Theory of Everything

Fast, cheap DNA sequencing technology now allows scientists to study unculturable microbes; the results are challenging some of biology's most fundamental notions. **By Alan Dove**

In the 19th century, some biologists began espousing an apparently absurd theory: that diseases were caused not by poor hygiene and foul vapors, as everyone knew they were, but by organisms too small to see with the naked eye. Pioneering researchers working on this strange idea developed sterile culture techniques, improved microscopes, and created other cutting-edge tools. Gradually, their results convinced their colleagues that the new germ theory of disease, odd as it seemed, was true.

In the 21st century, some biologists have begun espousing an even more absurd theory: that humans and other macroorganisms are not individual entities, as everyone knows they are, but complete ecosystems dependent on billions of microbes. Pioneering researchers working on this unusual idea have developed novel sampling strategies, powerful new gene sequencing and data analysis techniques, and other innovative technologies. Gradually, their results are convincing a new generation of scientists that the microbiomic theory of life, odd as it seems, may be true.

"There are more microbial genomes within us than we have human cells. We're a walking ecosystem. That's a pretty profound reality," says Timothy Harkins, director of research and development at **Life Technologies** in Carlsbad, California.

The Uncultured Majority

Microbiologists have long known that there are many bacteria, fungi, protozoans, and viruses that won't grow in the lab with current culturing techniques. Now the plummeting prices and skyrocketing sensitivity of next generation DNA sequencing technologies are finally letting researchers study this unculturable majority.

Most studies in this emerging field consist of sampling an environment, sequencing as much of the DNA in the sample as possible, and using the sequence information to identify the organisms in it and possibly their ecological functions. The results can be surprising. For example, microbiome analyses of the human gut have revealed that each person's large intestine carries a unique mix of bacterial species, and that pertur-

bations of this intestinal ecosystem may cause severe illness and even starvation.

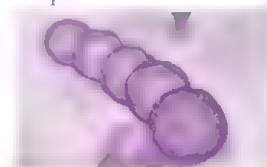
Though DNA sequencing forms the backbone of microbiomics, even the best sequencing protocols are useless without careful sampling and experimental design. "Sequencing is exciting and it's very interesting, and people have frequently ... said 'let's just sequence everything and sort it out later,'" says Jonathan Eisen, professor of evolution and ecology at the **University of California in Davis**, California. Eisen argues that this approach glosses over some crucial questions: "Do you want living cells? Do you want dead cells too? It's a pretty coarse tool to just say, 'I'm going to look at DNA.'"

Besides adding confounding factors such as dead cells and host DNA, poor sampling can destroy some of the genomes researchers wanted to find in the first place. "When [an anaerobic] bacterium is exposed to an oxygen environment it goes into apoptosis and kills itself, and shreds its genome, so how do you characterize something like that?" asks Harkins.

Once they've determined how to collect a useful sample, investigators need to decide exactly what questions they intend to ask, and how they want to frame the answers. Fortunately, zoologists and botanists have been studying ways to characterize ecosystems for decades. Unfortunately, they haven't reached much agreement. **continued>**

"There are more microbial genomes within us than we have human cells. We're a walking ecosystem. That's a pretty profound reality."

Streptococci



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To classify the organisms, biologists can take either a taxonomic approach, making a list of the species that are present and sorting them by their characteristics and the niches they occupy, or focus on constructing phylogenetic trees based on evolutionary relationships. Both methods have adherents and detractors. “People have been arguing about this for a hundred years,” says Eisen, a member of the phylogenetic camp.

Quantifying the diversity in ecosystems is somewhat more straightforward. Ecologists generally measure three types of diversity: alpha diversity, based on the number of species or phylogenetic groups in a specific area; beta diversity, which compares diversity between different areas; and gamma diversity, which uses alpha and beta to account for the total biodiversity of a large ecosystem. In medical microbiomics, researchers often measure the alpha diversity within a single person’s microbial sample, and calculate beta diversity between the microbiomes from different people.

Covering the Bases

After settling on an experimental design, microbiomics researchers move to the sequencing phase, where they face another major choice: sequencing ribosomal RNA (rRNA) or sequencing random snippets of whole genomes.

In rRNA sequencing, investigators use primers designed to amplify only the genes for 16S ribosomal RNA, a molecule that has changed very slowly throughout evolution. The number of different rRNA sequences in a sample is a good proxy for the number of species, and public databases of such sequences can be used to identify many organisms. Shotgun or metagenomic sequencing, in contrast, involves sequencing short, random pieces of all of the genomes in a sample, then trying to piece them together afterward.

Each method has advantages and drawbacks. “There are folks who like the shotgun approach I think because overall it can be easier, [but] the one thing the ribosomal RNA approach offers is [it’s effectively] an enrichment technique,” says Todd Arnold, head of research and development at 454 Life Sciences, a Roche Company in Branford, Connecticut. Ribosomal RNA sequencing is particularly useful for samples from human microbiomes, as the technique makes it relatively easy to ignore the enormous background of host DNA and focus only on the microbial components. Researchers in the field also regard rRNA sequencing

“What is a healthy microbiome? We don’t know yet really, we’re just scratching the surface.”

as the more mature technology, with better-defined procedures and clearer equipment choices.

However, metagenomic sequencing can identify a much wider range of variations across entire genomes, and may eventually enable scientists to sequence whole genomes in mixed samples. “I think there are aspects of metagenomic analysis which are becoming more routine, more off-the-shelf, [and] there’s a lot more that could be learned from metagenomic data,” says David Relman, professor of microbiology and immunology at Stanford University in Stanford, California.

Fortunately, sequencing equipment makers show no signs of resting on their laurels, and several companies are vying to increase their machines’ performance for both types of efforts. Though many microbiomics researchers have settled recently on Illumina’s HiSeq system for rRNA projects, Eisen is quick to point out that sequencing technology is still changing rapidly. “I don’t think it’s reached a plateau,” he says, adding that genome sequencing for metagenomics studies is particularly ripe for new breakthroughs.

Regardless of the platform they choose, biologists can expect the technology to be relatively user-friendly. “Sequencing is no longer seen as a skill for those who are very, very good at sequencing, it’s no longer in core labs,” says Arnold. Instead, modern high throughput sequencing machines are highly automated and include software that analyzes the raw data and performs initial quality control checks on it.

As a few early applications of microbial sequencing have reached the heavily regulated world of medicine and drug development, some gear makers have taken the automation a step further. For example, the Applied Biosystems Microseq platform performs traditional Sanger sequencing to identify bacterial and fungal contaminants in pharmaceutical manufacturing facilities. The system streamlines sequencing to detect a handful of specific organisms quickly and accurately, rather than probing the entire microbiome for all of the species present.

Down the Data Mine

Streamlined data and simple answers are the opposite of what basic researchers get from microbiome samples, though. “[Microbiomic] studies involve deep sequencing and are data-intensive—for both data storage and data analysis,” says Susan Knowles, senior marketing manager for microbiology at Illumina in San Diego, California. With the field still in its infancy, most of the software for analyzing those data comes from the investigators themselves. “There [is] a range of open source tools that researchers use for [microbiomics],” says Knowles, adding that “most of these data analysis tools require some bioinformatics skills.”

For scientists who are new to high throughput sequencing, the sheer quantity of information coming out of a sequencing machine can be a shock. 454’s Arnold says that the deluge of data often flummoxes newcomers.

In projects that focus on rRNA, investigators can take advantage of the simplified pool of possible sequences and large databases of known

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rRNA genes. Because this technique is more established than shotgun microbiome sequencing, the software for analyzing rRNA is also somewhat easier to use. Depending on the information experimenters hope to find, they may be able to complete a simple rRNA project without having to hire—or become—bioinformaticians.

Shotgun sequencing is a different story. “The methods to do that with metagenomics are much more complex,” says Eisen. In a metagenomics study, scientists have to identify putative genes from fragmentary sequences, determine what families those genes belong to, and try to identify what organisms they come from. Each step presents unique and serious challenges.

Microbiomic data analysis also raises a question that has vexed biologists for centuries: Exactly what is a species? Botanists and zoologists have reached some tentative definitions, but in bacteria, promiscuous gene swapping and rapid evolution make the concept trickier. For viruses, it may not apply at all. Worse, microbiomics itself could undermine traditional views of species distinctions. If each organism’s microbial ecosystem drives crucial parts of its biology, where does one individual end and the next begin?

To avoid being bogged down in philosophy, many microbiome researchers have settled on the idea of operational taxonomic units (OTUs), a practical, gene sequence-based analog of the species concept. Sequences that diverge beyond a certain threshold fall into distinct OTUs.

The European-funded MetaHIT project has recently used another approach: characterizing the human intestinal microbiome based on the putative functions of the different gene families in the sample, rather than by the species carrying those genes. Eisen explains that “they were some of the first people to do beta diversity and alpha diversity of function,” just as biologists have done with taxa. In this view, the organisms’ contributions to the ecosystem are what matter, not their identities or evolutionary origins.

After deciding how they want to view an ecosystem, researchers need to account for the inherent biases in their data. “There is no unbiased approach. The real challenge and important goal is to understand how biases arise in data, and what [one can] do to either minimize them or control for them,” says Relman. As examples, exposing samples to oxygen will eliminate obligate anaerobes, sequencing DNA will ignore RNA-containing viruses, and gentle extraction techniques may fail to lyse durable fungal or bacterial spores.

Going Long

While early pioneers in microbiomics continue sorting out the best ways to ask the scientific questions, engineers and equipment makers are trying to address some of the remaining technical needs. One of the top items on the agenda is longer sequence reads. “The longer the read the better,” says Harkin. He adds that “300 bases seems to be a good sweet spot, 300–350, and then the next sweet spot is when you get up to say 600, 800 base pairs.” Those lengths allow researchers to map microbial diversity at distinct levels of resolution, with greater lengths providing finer-grained separations between species.

Longer rRNA sequence reads allow researchers to distinguish organisms more clearly on a phylogenetic tree or taxonomic list, and longer metagenomic reads make it easier to assemble larger portions of each organism’s genome. Companies are also trying to make their sequencing systems more efficient at handling multiple samples, which is especially important for large clinical studies that try to identify variations in microbiomes across a human population.

Scientists are also trying to define clear methods and controls to ensure reproducible results. That’s turned out to be a thorny problem. “People have not tested a lot of the methods that are being used, they push a button and they run them, and we do the same thing,” says Eisen. Highly automated sequencing systems make it easy to produce results, but without clear guidelines for data analysis it’s unclear what those results mean. To try to establish a reference point, Eisen and his colleagues recently created a completely artificial microbiome by mixing known bacterial species that would never encounter each other in nature. “We shotgun sequenced them with different methods, and we tested how much we could actually figure out about a system [for which] we knew the answer. Even in that relatively simple artificial system some parts were very hard,” he says.

Classical microbiology may be able to help. Relman says that the new sequencing technologies, plus ongoing efforts to characterize bacterial environments in more detail, are enabling investigators to determine the culturing requirements for previously unculturable organisms. Growing these microbes in the lab makes it much easier to study them.

More exploratory surveys of microbiomes should also clarify some of the field’s boundaries, especially in medicine. For example, an ongoing project to study the lung microbiome has identified wide microbial population variations in the lungs of healthy individuals. “What is a healthy microbiome? We don’t know yet really, we’re just scratching the surface,” says Harkin.

Meanwhile, microbiome sequencing and data analysis continue getting simpler and cheaper, so that even undergraduates and nonscientists can now study microfauna almost as easily as fauna. “You’d be amazed at how many people are just starting to do this with ribosomal RNA. It’s a little bit different than what you might do with a set of binoculars and a bird book, but they get it,” says Eisen.

Alan Dove is a science writer and editor based in Massachusetts.

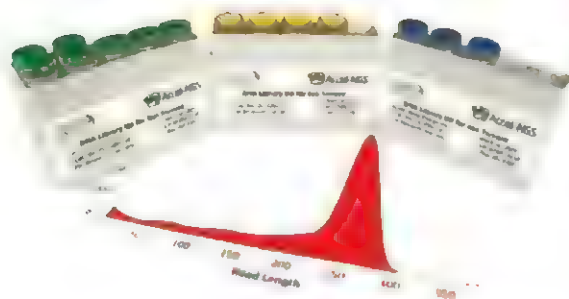
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Position Announcement Crop Physiologist

Crop Physiologist (OIE 002840-2013), Purdue University, Department of Agronomy, 915 West State Street, West Lafayette, IN 47907-2054. Tenure-track faculty position. Assistant or Associate Professor (academic 10-month appointment will include research (majority) and teaching).

Responsibilities: The successful candidate will conduct innovative crop physiology research aimed at enhancing plant productivity and environmental sustainability for maize or soybean. The research context is broad, and could include abiotic stress tolerance, input use efficiency (e.g., nutrients, water), and plant resiliency to climate change. Candidates would ideally be capable of employing creative technologies in plant phenotyping or modeling for achieving advanced understanding/determination of important physiological traits. The successful candidate will establish an externally funded and internationally recognized research program, teach relevant courses in the crop science curriculum, and advise graduate students. **Qualifications:** A Ph.D. in crop physiology, plant physiology, or a related discipline is required. Doctoral and/or postdoctoral research experience aimed at understanding impacts of genotype, environment, and/or management interactions on physiological responses of crop species is highly desirable. Commitment to teaching excellence is important. The ability to work as part of a team, and the ability to interact with students, government agencies, industry, and the general public are essential. Successful candidates must be committed to fostering diversity. Candidates with experience in, or the desire to develop, international dimensions of the discipline are preferred. The College of Agriculture at Purdue University is deeply committed to the three land-grant missions (teaching, research, and Extension), to international activities and perspectives that span all missions, and to advancing diversity in all areas of faculty effort. Outstanding opportunities for interdisciplinary research exist within the Purdue University community. The College has 11 academic departments, 300 faculty, 2650 undergraduate students, and 685 graduate students. The College's strategic plan can be accessed at <http://www.ag.purdue.edu/Pages/strategicplan.aspx>.

Applications: Review of applications will begin **August 15, 2013** and will continue until the position is filled. A background check will be required for employment for this position. Candidates submit a letter of application, statements describing their research and teaching interests, official transcripts, names of three individuals that can serve as references, and a detailed curriculum vita that includes education, experience, additional qualifications, and publications to: **Tony J. Vyn, Chair of the Search Committee (c/o Lisa Green, lgreen06@purdue.edu), Department of Agronomy, Purdue University, 915 W. State Street, West Lafayette, IN 47907-2054, Phone: (765) 496-3757; Fax (765) 496-2926, Email: tvyn@purdue.edu, URL: <http://www.ag.purdue.edu/agry/>.**

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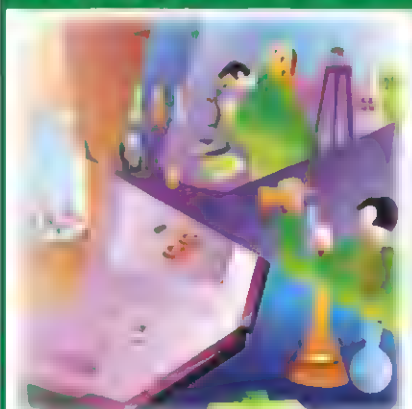
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NYU NEUROSCIENCE

Valentino D.B. Mazzia MD, JD Professorship in Anesthesiology and Neuroscience

The Department of Anesthesiology and the Neuroscience Institute of NYU Langone Medical Center are recruiting an outstanding MD/PhD or MD neuroscientist to become tenured Professor and Vice Chair of Research for the Department of Anesthesiology and a member of the Neuroscience Institute at NYU. Applicants should have an ongoing extramurally supported research program in any area of neuroscience related to anesthesiology, such as ion channel physiology, neuronal plasticity, mechanisms of pain, consciousness and higher cognitive function, etc. The candidate will be responsible for their own research program and expected to grow basic and clinical neuroscience research in the Department of Anesthesiology and to mentor junior faculty, residents, and students.

Please submit your CV with a statement describing your scientific and mentoring interests by email to:

Thomas.Blanch@nyumc.org
or to

Richard.Tsien@nyumc.org

Submission deadline: **May 30, 2013**

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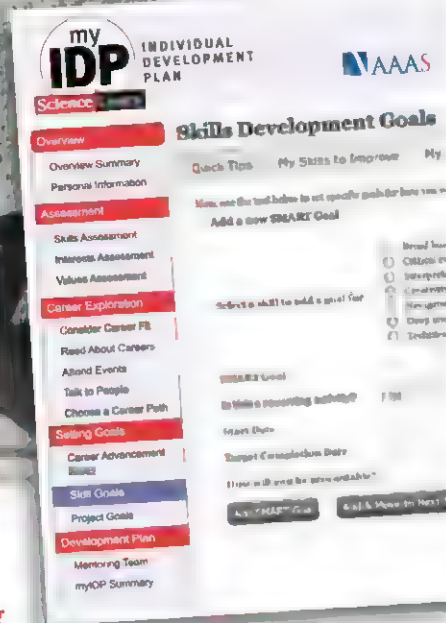
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W. Harry Feinstone Department of Molecular Microbiology and Immunology Alfred and Jill Sommer Professor and Chair

The Johns Hopkins Bloomberg School of Public Health invites applications for Professor and Chair of the Department of Molecular Microbiology and Immunology. The successful applicant will have an outstanding record of academic and research accomplishment and demonstrated leadership and administrative abilities.

The mission of the Department is to advance understanding of basic biological processes underlying the major infectious diseases affecting public health. To combat global health threats caused by parasites (malaria), bacteria (tuberculosis) and viruses (AIDS), faculty and students conduct their studies in laboratories, in the field and in clinics. The Department has 50 full-time faculty, 100 doctoral and master's students and a diverse research program. The Bloomberg School of Public Health has ten departments and more than 500 full-time faculty and 2,000 graduate-level students. The School is located on the Johns Hopkins University East Baltimore medical campus, a collaborative and highly interactive environment with a superb research infrastructure.

The Johns Hopkins University is committed to recruiting, supporting and fostering a diverse community of outstanding faculty, staff and students. Women and under-represented minority candidates are particularly encouraged to apply. EEO/AA.

Review of candidates will begin in **July 2013**. Applications should include a Curriculum Vitae and statement of research interest and vision of leadership. Submit to:

Chair, Search Committee, c/o Ms. Susan Williams
Office of the Dean
Johns Hopkins Bloomberg School of Public Health
615 N. Wolfe St., Room W1041, Baltimore, MD 21205
suwillia@jhsph.edu

GRANTS



6th Yamada Research Grant (Japan)

The Yamada Research Grant aims to promote the latest and highly original studies from multiple points of view in the fields of preventive medicine with bee products and natural products.

1. Eligible researchers:

- 1) New research grant: For researchers or beekeepers who have never received the research grant from the Yamada Research Grant.
- 2) Continuing research grant: For researchers or co-researchers who have previously received a research grant from the Yamada Research Grant.

2. Subject for research:

- 1) New research grant:
 - (1) Preventive medical research. The following fields will be given a priority.
 - A. Brain science, neuroscience, mental science (dementia, depression, etc.)
 - B. Cardiology, gastroenterology, metabolomics (metabolic syndrome, intestinal disease, etc.)
 - C. Immunology (cancer, allergy, infectious disease, gut immunity, etc.)
 - D. Otorhinolaryngology, ophthalmology, stomatology
 - E. Anti-aging medicine (skin, derma, bones, muscles, etc.) or longevity
 - (2) Research on environmental protection (including honey bee science, reconstruction support, etc.)
- 2) Continuing research grant

Progressive research of the theme done with the support of the Yamada Research Grant.

3. Grant amount:

- 1) New research grant: Up to 2,000,000 Japanese Yen per theme.
- 2) Continuing research grant: Up to 10,000,000 Japanese Yen per theme.

4. Language: English or Japanese

5. How to apply: Applications should be filled out the specified format on the following website according to application guideline for this grant.

The official website of Application for Yamada Research Grant 2013:
http://grant.bee-lab.jp/grant/grant_2013/guideline_eng.html

6. Application deadline: May 31st, 2013 (until 17:00 Japan Time)

How to inquire: If there are any questions, please contact the secretariat of this grant by e-mail. E-mail address: research-grant@yamada-bee.com

Open Rank Faculty (Professorial) - Translational Neuroscience (Three Positions)

The City College of New York

Three open-rank faculty (Professorial) positions in Translational Neuroscience are available at The City College of the City University of New York for researchers with MD, PhD, combined MD PhD or equivalent degree in Neuroscience or related disciplines

1. Clinical neuroscience researcher with a focus on neurological and/or psychiatric diseases (e.g., neurodegenerative diseases, psychiatric disorders, stroke, traumatic brain/spinal cord injuries) and expertise in brain stimulation and/or imaging techniques (e.g., TMS, tDCS, DBS, functional and structural MRI, PET)
2. Basic neuroscience researcher specializing in advanced techniques in brain stimulation and/or imaging (e.g., ultrasound stimulation, optogenetic stimulation, magnetic resonance, and in general, techniques still in early phases of clinical testing or pre-clinical development) for application in clinical settings
3. Computational neuroscience researcher with interests in normal brain function (e.g., sensory systems, sleep, consciousness, neuroeconomics) and/or neurological/psychiatric dysfunction (e.g., schizophrenia, autism, attention-deficit hyperactivity disorders). Significant experience in modeling and computational techniques is required

The successful candidates are expected to maintain an active, externally funded collaborative program of research, to publish in top-tier journals, and to be committed to both undergraduate and graduate education. A history of obtaining external grant support is necessary for senior candidates, junior candidates are expected to have a strong potential for funding. The optimal candidates should also have great interaction skills to foster research partnership with colleagues at CCNY



To apply, please view the original posting (Job ID 8049)
at www.cuny.edu and follow all instructions.

EO/AA Employer



ASSISTANT PROFESSOR Division of Immunology Department of Microbiology and Immunobiology Harvard Medical School, Boston, MA

The Division of Immunology of the Department of Microbiology and Immunobiology at Harvard Medical School invites applications for a tenure-track position at the rank of Assistant Professor. We seek an outstanding scientist performing basic and/or translational research at the interface of Immunology and Microbiology. Examples of preferred research areas include (but are not limited to): the innate/adaptive immune system interface, host/pathogen interactions, anti-microbial responses, and immune system microbiome interplay.

This position offers outstanding scholarly and scientific resources in a collegial and collaborative department with strong ties to related departments throughout Harvard University, the Harvard affiliated teaching hospitals and the Boston immunology/microbiology community. The position provides the opportunity to join a growing coalition of researchers at Harvard Medical School interested in molecular and quantitative approaches to basic immunological and microbiological diseases. The position also offers the opportunity to teach exceptional graduate and medical students with strong interests in immunology/microbiology. The research space will be located in a recently constructed research building at Harvard Medical School. Candidates must have a Ph.D., M.D. or an equivalent graduate degree.

For consideration please upload to the website below a cover letter, a C.V. and a concise summary of research accomplishments and interests which should include a list of publications. Contact information for three letters of reference should also be included.

The application period closes **July 31, 2013**.

Submit all materials via:

<https://academicpositions.harvard.edu/postings/4783>

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Max Planck Institute of Immunobiology and Epigenetics

Max Planck Institut für Immunbiologie und Epigenetik



Search for a new DIRECTOR

The Max Planck Institute of Immunobiology and Epigenetics in Freiburg (Germany) (www.ie-freiburg.mpg.de) is in the process of identifying a new director in the field of immunobiology.

The general interest of the institute is in mechanisms underlying the function of the immune system, cell signalling, regulation of gene expression and the epigenetic aspects of chromatin structure and function. We study these processes at the molecular, cellular and organismic levels and welcome the use of any experimental system.

The working language at the institute is English. Although there are no teaching obligations, the institute has close connections to the University of Freiburg.

The Max Planck Society is an independent, non-profit organization that promotes research at its own institutes. The Max Planck Society encourages the pursuit of new challenging directions that require the long-term commitment of substantial resources.

Qualified candidates should send a curriculum vitae, a short statement of research interests and scientific goals and reprints of key publications to the **Managing Director, Max Planck Institute of Immunobiology and Epigenetics, Stübeweg 51, 79108 Freiburg, Germany, before June 30th, 2013** (e-mail: jenuwein@ie-freiburg.mpg.de).

The Max Planck Society is aiming at increasing the percentage of women among its scientific leadership, particularly at the director level and therefore strongly encourages expressions of interest from and nominations of qualified women.



The University of Texas at Dallas The School of Natural Sciences and Mathematics

FACULTY POSITION IN BIOLOGICAL OR BIOMEDICAL PHYSICS

The Department of Physics at The University of Texas at Dallas (UTD) invites applications for a full-time tenure-track or tenured faculty position in the general areas of experimental biological physics, bio-materials physics, bio-medical physics, and advanced physical imaging methods, to begin sometime during the 2013-2014 academic year. A Ph.D. in physics is preferred, and a strong record of research experience in these or closely related fields is required. The successful candidate is expected to define and lead a cutting-edge research program and to develop research collaborations with other UTD programs in biological and bio-medical science and engineering, such as the Department of Molecular and Cell biology, the Department of Bioengineering, the Texas Biomedical Device Center, as well as with the nearby University of Texas Southwest Medical Center. Responsibilities also include teaching physics courses and supervising students at the undergraduate non-major, undergraduate major, and graduate levels. An appropriate amount of start-up funding and space will be available. UTD is primarily searching for someone at the tenure-track Assistant Professor level, but unusually well qualified candidates may be considered for appointment as Associate Professor or Professor.

Applications will be accepted and reviewed until the position is filled. Questions regarding this position may be directed to **Prof. Mark Lee** (marklee@utdallas.edu). Indication of gender and ethnicity for affirmative action statistical purposes is requested as part of the application.

Applicants should submit a cover letter, a curriculum vitae including publication list, a statement of research and teaching interests, and a minimum of three letters of recommendation via the ONLINE APPLICATION FORM available at: <http://go.utdallas.edu/pns130424>

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Every day Project 2061 staff use their expertise as teachers, researchers, and scientists to evaluate textbooks and assessments, create conceptual strand maps for educators, produce groundbreaking research and innovative books, CD-ROMs, and professional development workshops for educators, all in the service of achieving our goal of universal science literacy.

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POSITIONS OPEN

FACULTY POSITION

**Assistant/Associate/Full Professor in
Pharmaceutics**

The Department of Pharmaceutical Sciences in the College of Pharmacy at the University of Tennessee Health Science Center in Memphis, Tennessee, is seeking an outstanding applicant for a 12-month full-time, tenure-track faculty position at the full, associate, or assistant professor level that is state supported. The successful candidate is expected to have a strong research program in the area of: pharmaceutics, physical pharmacy, drug delivery, gene therapy, nanotechnology, nanomedicine, regenerative medicine, bio-imaging, biosensors, biomedical engineering, or other related discipline with focus on drug discovery and development. The successful candidate is expected to have a Ph.D. or equivalent degree, the ability to maintain a competitive extramural funding, a commitment to excellence in teaching Pharm.D. and graduate programs, and have excellent oral and written communication skills. Applications will be processed until the position is filled. Please submit a cover letter, curriculum vitae, summary of research and teaching interests, and three letters of reference to: **Dr. John Buolamwini, Chair of Faculty Search Committee, Department of Pharmaceutical Sciences, 847 Monroe Ave, Suite 327, Memphis, TN 38163**, electronically at e-mail: jbuolamwini@uthsc.edu.

The University of Tennessee Health Science Center is located in Memphis, TN, an economically vibrant center, with a metropolitan population of more than 1.3 million, reflecting the richness along the bluffs of the mighty Mississippi River. The College of Pharmacy is located in a new, six-story 187,000 square-foot building with state-of-the-art research space and teaching facility on the Health Science Center complex.

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